Bhattacharyya, T (2015) Parasite diversity and innovative serology: development of Trypanosoma cruzi lineagespecific diagnosis of Chagas disease and of prognostic assays for visceral leishmaniasis. PhD thesis, London School of Hygiene & Tropical Medicine. DOI: https://doi.org/10.17037/PUBS.0217366

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Parasite diversity and innovative serology: development of *Trypanosoma cruzi* lineage-specific diagnosis of Chagas disease and of prognostic assays for visceral leishmaniasis.

Tapan BHATTACHARYYA

Thesis submitted in accordance with the requirements for the degree of

Doctor of Philosophy of the University of London

November 2014

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LONDON SCHOOL OF HYGIENE & TROPICAL MEDICINE,
UNIVERSITY OF LONDON

Funded by European Commission Seventh Framework Programmes for Research:
'ChagasEpiNet' and 'NIDIAG'

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I, Tapan Bhattacharyya, confirm that the work presented in this thesis is my own.

Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Tapan BHATTACHARYYA

November 2014

ABSTRACT

Trypanosoma cruzi and the *Leishmania donovani* complex are parasitic protozoa that, respectively, cause Chagas disease in the Americas, and visceral leishmaniasis, predominantly in South Asia, East Africa, and Brazil.

T. cruzi is divided into the lineages TcI-TcVI. The relationship between infecting lineage(s) and spectrum of clinical presentations remains poorly understood. This project developed lineage-specific serology to identify an individual's history of lineage infection. A high level of polymorphism in the surface mucin TSSA was identified, and lineage-specific synthetic peptides based on this diversity were applied here in ELISA with chagasic sera from endemic countries. Peptide TSSApep-II/V/VI, based on a sequence common to those lineages, was widely recognised by sera from Southern Cone countries, and also unexpectedly by four samples from Ecuador; TSSApep-V/VI, which differs by a single amino acid from TSSApep-II/V/VI, was also recognised in these regions. A single TSSApep-IV reaction was seen in both Colombia and Venezuela. However, TSSApep-I was rarely and weakly recognised among the serum panel. Among the Brazilian patients, a much higher proportion of TSSApep-II/V/VI responders had ECG abnormailities than non-responders (38% vs. 17%, $p<0.0001$).

Rapid diagnostic tests for *L. donovani* complex infection based on rK39 antigen have lower sensitivity in East Africa compared to South Asia. The homologous sequences of rK39, and of another proposed diagnostic antigen HASPB, were amplified from a panel of East African *L. donovani* strains, and compared to published sequences, revealing significant diversity from rK39 and South Asian sequences, and non-canonical combinations of HASPB repeats. Cohorts of Indian and Sudanese VL patients were assayed by ELISA for anti-*Leishmania* IgG levels. There was an overall 46.8 – 61.7 fold lower response in the Sudanese cohort, as calculated by mean reciprocal $\log_{10}t_{50}$ titres, regardless of antigen source, patient gender or age.

An investigation into the association of IgG subclass reactivity with VL clinical status revealed significantly elevated IgG1 levels in patients with active (pre-treatment) VL and those with post-therapy relapse compared to those deemed to be cured. A novel prototype

rapid immunochromatographic test to detect IgG1 gave > 80% of relapsed VL patients as IgG1 positive, and 80% of cured patients as IgG1 negative.

ACKNOWLEDGEMENTS - General

The research presented in this thesis was supported by the EC Framework Programme Projects 'Comparative epidemiology of genetic lineages of *Trypanosoma cruzi*' (**ChagasEpiNet**, co-ordinator Prof MA Miles, LSHTM), and 'Syndromic approach to neglected infectious diseases at primary health care level: an international collaboration on integrated diagnosis-treatment platforms' (**NIDIAG**, co-ordinator Prof M Boelaert, ITG).

In 2009, the centenary of the discoveries of Carlos Chagas, I had the great privilege to begin my doctoral research with, and receive the mentoring of, **Prof. Michael A. Miles**, Faculty of Infectious & Tropical Diseases, LSHTM. I have benefited greatly from expertise, reagents, technologies, and support provided from many colleagues:

Miles group, LSHTM Nidia Acosta, Armon Ayandeh, Rania Baleela, Duncan Bowes, Andrew Falconar, Sinead Fitzpatrick, Michael Gaunt, Marissa Gripenberg, Hannah Hafezi, Kiera Hayes, Osama Idris, Michael Lewis, Martin Llewellyn, Tegwen Marlais, Isabel Mauricio, Louisa Messenger, Emily Mills, Sara Silva Pereira, Trang Tran, Matthew Yeo.

ChagasEpiNet partners Prof Björn Andersson, Oscar Franzén (Karolinska Institutet, Sweden); Christian Barnabé (GEMI-IDR, France); Prof Hernan Carrasco, Maikell Segovia (Universidad Central de Venezuela); Patricio Diosque (Universidad Nacional de Salta, Argentina); Prof Alejandro Luquetti, Suelene Tavares, Liliane Siriano, Christian Rodrigues, Gabi Miranda, Rosângela Amaral (Hospital das Clinicas, Federal University of Goias, Brazil); Prof Ana Jansen, Fabiana Rocha, Samanta Xavier, Juliana Barros, André Roque, André Perreira (FIOCruz, Rio de Janeiro, Brazil); Prof Yves Carlier, Sabrina Cencig (Université Libre de Bruxelles, Belgium); Prof Mario Grijalva, Jaime Costales (Pontificia Universidad Católica del Ecuador); Prof Felipe Guhl, Juan-David Ramirez (Universidad de los Andes, Colombia); Lineth Garcia (Bolivia).

NIDIAG partners Prof Marleen Boelaert (Instituut voor Tropische Geneeskunde, Belgium), Prof Sayda El-Safi, Dr Osman Ahmed, Dr Osama Eisa, Dr A Saad (University of Khartoum, Sudan); Prof Shyam Sundar, Om Prakash Singh, Rajiv Srivastava, Dinesh Prajapati (Banaras

Hindu University, India); Pascal Mertens, Caroline Thunissen (Coris BioConcept, Belgium); Prof Suman Rijal, Narayan Bhattarai, Keshav Rai (BP Koirala Institiue of Health Sciences, Dharan, Nepal).

LSHTM Ozan Gundogdu, Eloise Thompson, Carolyn Stanley, Keith Flanders.

External Carolyn Hyde (University College London); Sergey Litvinov (Aptum Biologicals).

ACKNOWLEDGEMENTS – Specific contributions of others to the work described herein

Production of synthetic peptides, experimental design & analysis, comments/editing of manuscripts: Andrew Falconar

Access to chagasic and control sera: Prof Alejandro Luquetti, Prof Mario Grijalva, Jaime Costales, Prof Felipe Guhl, Juan-David Ramirez, Lineth Garcia, Prof Hernan Carrasco, Patricio Diosque

Supply of *T. cruzi* strains and/or DNA: Michael Lewis, Louisa Messenger, Martin Llewellyn, Cristian Barnabe

Assisted phylogenetic analyses of sequences generated by candidate: Louisa Messenger, Michael Lewis, Matthew Yeo

Novel bioinformatic tools: Sergey Litvinov

Access to visceral leishmaniasis and control sera: Prof Shyam Sundar, Om Prakash Singh, Rajiv Srivastava, Dinesh Prajapati, Prof Sayda El-Safi, Dr Osman Ahemd, Dr A Saad

Production of IgG1 immunochromatographic rapid diagnostic test: Pascal Mertens, Caroline Thunissen

Students performing research work under the candidate's supervision: Duncan Bowes, Armon Ayandeh, Marissa Gripenberg, Trang Tran, Sara Silva Pereira.

CONTENTS

ABBREVIATIONS

αMEM	Minimum essential medium Eagle, α modification
aa	amino acid
AFLP	Amplified fragment length polymorphism
AMCHA	*Iniciativa de Vigilancia y Prevención de la Enfermedad de Chagas en la Amazonía* (Initiative for Surveillance and Control of Chagas Disease in the Amazon Region)
BENEFIT	Benznidazole Evaluation For Interrupting Trypanosomiasis
BLAST	Basic local alignment search tool
bp	Base pair(s)
CDC	Centers for Disease Control, USA
CI	confidence interval
DALY	Disability- adjusted life years
DAT	Direct agglutination test
ddH_2O	Double-distilled water
DDT	dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DTU	Discrete typing unit
EDTA	Ethylenediaminetetraacetic acid disodium salt $C_{10}H_{14}N_2Na_2O_8$
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide $C_{21}H_{20}BrN_3$
EtOH	Ethanol C_2H_5OH
FBS	Foetal bovine serum
H_2O_2	Hydrogen peroxide
HASPB	Hydrophilic acylated surface protein B
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt

HRP	Horse radish peroxidase
ICT	Immunochromatographic test
IIF	Indirect immunofluorescence assay
IFAT	Indirect fluorescent antibody Test
Ig	Immunoglobulin
IHA	Indirect hemagglutination assay
IL-10	Interleukin 10
INCOSUR	*Iniciativa del Cono Sur para Control/Eliminación de Chagas* (Southern Cone Initiative for Control/Elimination of Chagas Disease)
IPA	*Iniciativa de los Países Andinos* (Initiative of the Andean Countries)
IPCA	Iniciativa de los Países de Centro América para la Interrupción de la Transmisión Vectorial y Transfusional de la Enfermedad de Chagas (Initiative of the Central American Countries for the Interruption of the Vectorial and Transfusional Transmission of Chagas Disease)
kb	Kilobase pairs
LSHTM	London School of Hygiene and Tropical Medicine
Mb	Megabase pairs
MLEE	Multilocus enzyme electrophoresis
MLMT	Multilocus microsatellite typing
MLST	Multilocus sequence typing
MSF	Meédicins Sans Frontières
mya	Million years ago
nt	Nucleotide(s)
ORF	Open reading frame
PAHO	Pan-American Health Organisation
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PKDL	Post kala-azar dermal leishmaniasis
rDNA	ribosomal DNA
RDT	Rapid diagnostic test

RFLP	Restriction fragment length polymorphism
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal RNA
SNP	Single nucleotide polymorphism
TAE	Tris-Acetic acid-EDTA buffer
TSSA	Trypomastigote small surface antigen
UV	Ultra-violet light
VL	Visceral leishmaniasis
WHO	World Health Organisation
WHO/TDR	World Health Organisation Special Programme for Research and Training in Tropical Diseases

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1. GENERAL INTRODUCTION

1.1 *Trypanosoma cruzi* and Chagas disease

Chagas disease (American trypanosomiasis) remains an important parasitic disease in the Americas. The aetiological agent, the trypanosomatid protozoan *Trypanosoma cruzi*, is transmitted by species of triatomine bugs and is estimated to infect at least 8 million people in the endemic region (www.who.int/mediacentre/news/releases/2007/pr36/en/) (Figure 1).

Figure 1 Distribution of Chagas disease. Endemic region indicated in purple (Morel & Lazdins, 2003).

Early symptoms may include fever, fatigue and generalised oedema, but chronic infection can lead to debilitation and death by cardiac and/or intestinal complications. The estimated disease burden in endemic countries is over 0.6 million DALYs, with around 14.000 deaths per annum (Hotez *et al.*, 2009). Genetically diverse, *T. cruzi* is divided into the intra-species lineages TcI-TcVI. The association of genotype with geographical distribution is complex: TcI is found from the USA to Argentina in sylvatic cycles of transmission, with domestic infection principally north of the Amazon; TcII is found predominantly in human infections in the Southern Cone countries, and TcV and TcVI have only, as yet, been reported in Southern Cone countries. The different genotypes may also be associated with different natural transmission cycles (*i.e.,* the insect vectors and mammalian hosts in sylvatic and domestic situations), and the varying symptoms of the disease. A key goal remains the clarification of this latter relationship. Despite recent successes in disrupting the vector-borne transmission cycle in the Southern Cone countries, no prophylactic vaccine or

satisfactory chemotherapy exists for Chagas disease, especially in the chronic phase. It threatens to emerge as a public health issue beyond the endemic region, where chronically-infected migrants are a source for parasite transmission.

1.2 *Leishmania donovani* complex and visceral leishmaniasis

The human leishmaniases exhibit a broad range of clinical syndromes, including: localised self-healing skin lesions (cutaneous leishmaniasis, CL); nasopharyngeal tissue destruction (mucocutaneous leishmaniasis, MCL); hepatosplenomegaly (visceral leishmaniasis, VL, 'kala-azar'); post-kala-azar dermal leishmaniasis (PKDL, a sequel of VL). The different diseases are caused by infection by various species of the trypanosomatid protozoan *Leishmania* genus, acquired during bloodmeal feeding by an infected sandfly vector. The gravest form is VL; symptomatic disease is usually fatal if untreated. The great majority of the estimated 200,000 to 400,000 annual new VL cases are found in South Asia, East Africa, and Brazil (Figure 2), with 50,000 deaths per year (WHO, 2010a; Alvar *et al*., 2012).

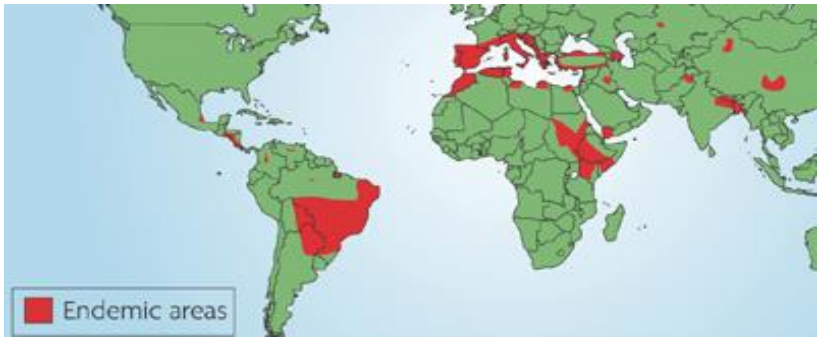


Figure 2 Distribution of VL. (Chappuis *et al.,* 2007)

VL is caused by species of the *L. donovani* complex. These are *L. donovani* in East Africa and South Asia, and in Latin America *L. infantum* (historically '*L. chagasi')* imported from Europe in recent centuries. Populations most at risk are among the poorest of society, and also non-immune migrants seeking refuge from war or famine in endemic areas. Epidemics have been particularly catastrophic. The rise of HIV infection has also been followed by an increase in incidence in VL cases worldwide. No prophylactic human vaccine exists, and diagnosis is by clinical signs, demonstration of intracellular amastigote forms, or

serology. Rapid diagnostic tests based on serology (detection of presence of anti-leishmanial antibodies) have proved very sensitive in certain endemic regions, but less so in others, for reasons that hither to have been unclear.

2. LITERATURE REVIEW

2.1 Carlos Chagas and the discovery of *T. cruzi*

Carlos Chagas (1879-1934), a doctor working in Lassance, southern-central Brazil, observed that protozoan flagellates found in faeces of night-biting triatomine bugs caused experimentally infected marmosets to develop a circulating trypanosome. Originally naming them *Schizotrypanum cruzi*, he went on to find the parasites circulating in children in Lassance with an acute febrile illness (Figure 3). Subsequently, he described the life cycle and mammalian host (Chagas, 1909). Emile Brumpt (1877-1951) later showed the mechanism of parasite transmission to be through infected bug faeces.

Figure 3 Carlos Chagas examining baby. Lassance, Brazil c.1910 (Arquivo Iconográfico da Casa de Oswaldo Cruz, Brazil*).*

2.2 *T. cruzi*

2.2.1 Taxonomy

The taxonomy of *T. cruzi* is given in Figure 4. The full taxonomic designation *Trypanosoma (Schizotrypanum) cruzi* refers to those trypanosomes of the subgenus *Schizotrypanum*, which undergo intracellular multiplication in vertebrates.

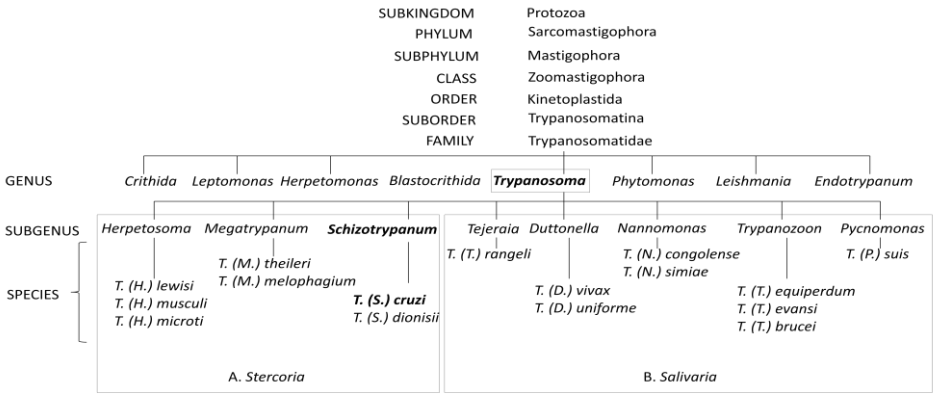


Figure 4 Taxonomy of *T. cruzi*. (WHO, 2002)

2.2.2 Life cycle

The life cycle of *T. cruzi* is summarised in Figure 5.

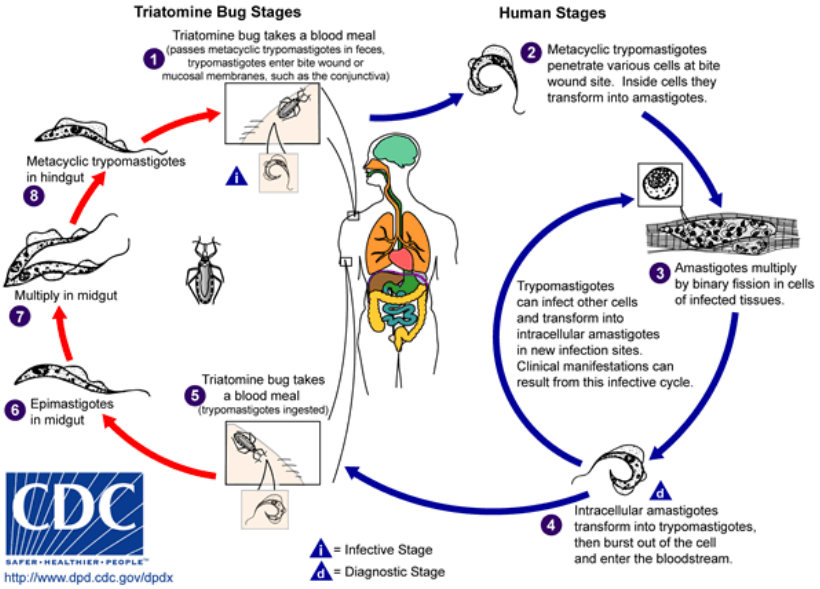


Figure 5 Life cycle of *T. cruzi* (CDC).

After an infected triatomine takes a blood meal, infective metacyclic trypomastigotes deposited in its faeces enter the host through the bite wound, abraded skin, or intact mucosal membranes, *e.g.*, conjunctiva, invading host cells near the inoculation site. Differentiation to intracellular amastigotes occurs. After cycles of binary replication, doubling every ~12 hrs, the amastigotes differentiate into trypomastigotes, rupture the host cell, releasing this form into the bloodstream, where they can infect various cell types and transform into intracellular amastigotes in new sites. Circulating trypomastigotes, ingested during a bloodmeal on an infected host by the triatomine, transform into epimastigotes in the vector's midgut, where they multiply before differentiating into infective metacyclic trypomastigotes in the hindgut (Figure 6). These are eliminated with triatomine faeces and are able to infect the vertebrate host.

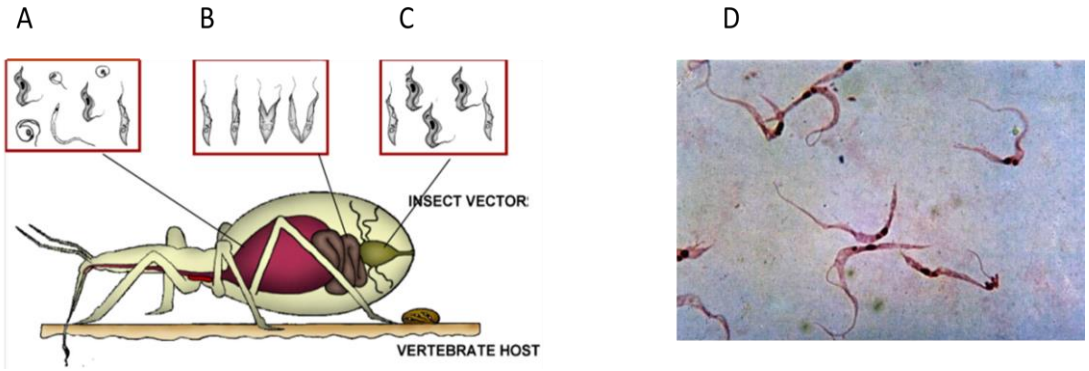


Figure 6 *T cruzi* development in the triatomine vector. [A] Ingested epimastigotes and some sphaeromastigotes in stomach. [B] Epimastigotes multiply in midgut. [C] Transformation into metacyclic trypomastigotes in rectum. (Garcia *et al*., 2007) [D]. Epimastigotes.

2.2.3 Ultrastructure

A schematic of the epimastigote form of *T. cruzi* is shown in Figure 7. The single mitochondrion, which contains the kinetoplast, drives the flagellum which stems from it and extends beyond the cell. Figures 7A and 7B depict the epimastigote and trypomastigote forms respectively. The kinetoplast contains extranuclear DNA arranged in mini- and

maxicircles. The flagellum has nine pairs of peripheral microtubule doublets surrounding a central pair (Figure 7C).

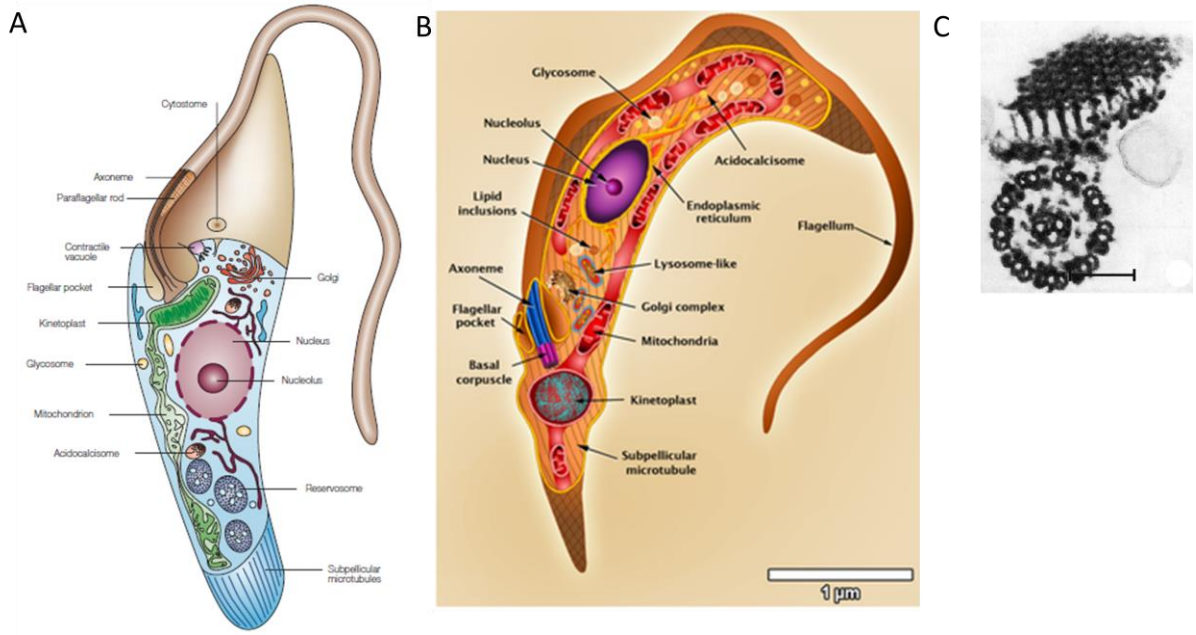


Figure 7 *T. cruzi* ultrastucture. [A] Schematic of epimastigote form. (Docampo *et al*., 2005) [B] Trypomastigote has centrally positioned nucleus, and kinetoplast located towards the posterior (Teixeira *et al*., 2012). [C] Transversal view of thin section of flagellum of *T. cruzi*. Bar = 50 nm. (de Souza, 2009)

2.2.4 Vertebrate host cell invasion

Host cell invasion by *T. cruzi* is a multi-step process involving interaction of parasite and host cell-surface molecules. *In vitro* cultured trypomastigotes express surface glycoproteins of the gp85 family, which have extracellular matrix component binding capability (Yoshida, 2006). These allow the subsequent enzymatic degradation of the matrix surrounding the target host cell, enabling parasite surface molecules such as trans-sialidase

(Butler *et al*., 2013) to interact with, and initiate the invasion of, the host cell. Host proteins identified as interacting with *T. cruzi* include kinin receptor (Todorov *et al*., 2003) and the nerve growth factor receptor TrKa (Melo-Jorge & Pereira, 2007).

The trypomastigote becomes stably attached to the host cell, and initiates a signaling process leading to an elevation of intracellular free Ca^{2+}. Host cell lysosomes are recruited to migrate to the trypomastigote attachment site, fuse with the plasma membrane, and allow the parasite to enter the host cell in via a parasitophorous vacuole formed by lysosomal membranes. *T. cruzi*-induced Low Density Lipoprotein receptor (LDLr) upregulation in infected cells has been shown, the same paper also reporting the role of parasite-bound LDLr in lysosome trafficking and fusion to the parasitophorus vacuole (Nagajyothi *et al.* 2011). The acid environment of the parasitophorous vacuole favours the activity of Tc-Tox, the *T. cruzi* pore-forming molecule, leading to vacuole disruption. The acidic pH also stimulates trypomastigote differentiation into amastigotes, the intracellular replicative forms. After these differentiate into trypomastigotes, the host cell is ruptured, and this infective stage is released into the bloodstream. (Tan & Andrews, 2002; Manoel-Caetano & Silva, 2007)

2.2.5 Vectors

Several triatomine bug species are particularly important in human *T. cruzi* transmission, due to their ability to colonise human habitations and peri-domestic areas. Examples of these, along with geographic distributions, are shown in Figure 8.

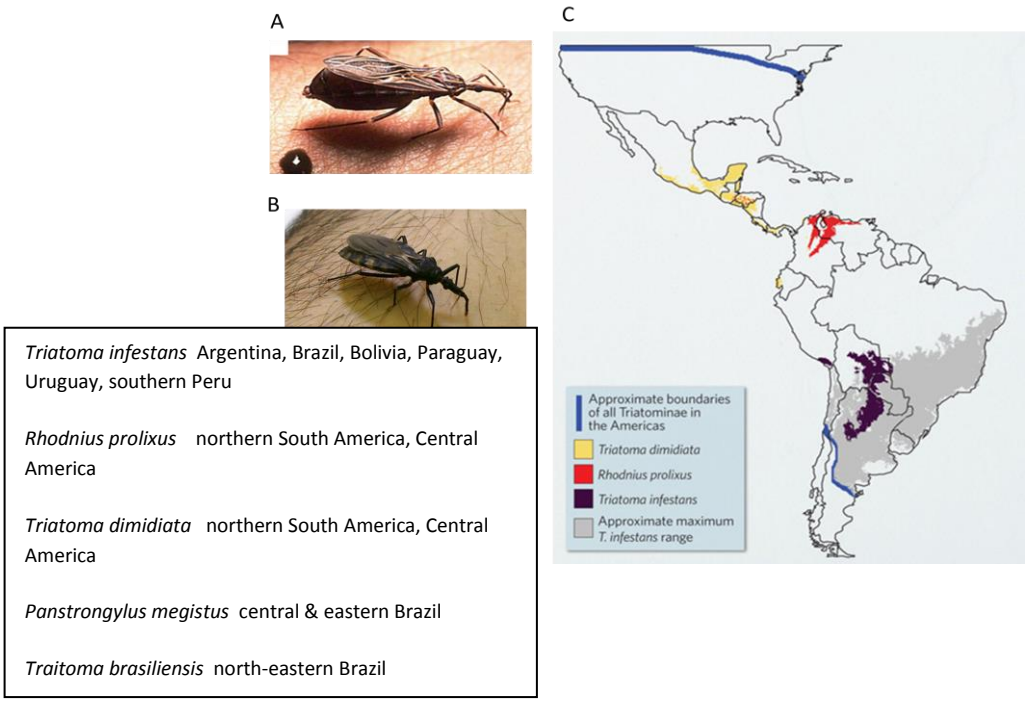


Figure 8 Triatomine vectors of *T. cruzi*. [A] *R. prolixus* taking blood meal through human skin. Deposited faeces can be seen. (WHO/TDR/Stammers). [B] *T. infestans*. (photo credit T. Marlais, LSHTM). [C] Distribution in the Americas. (nature.com/nature/journal/v465/n7301_supp/box/nature09222_BX2.html). INSET: Miles *et al*., 2009b

Both sexes are obligate haematophages, requiring at least one blood meal prior to progression to the next of their five growth instars. The bugs are often found in domestic or peri-domestic environments, where the poor nature of the dwelling's construction favours colonisation by triatomines (Figure 9). Triatomine species emerge from their sanctuaries in such dwellings to take a blood meal from the human inhabitants at night.

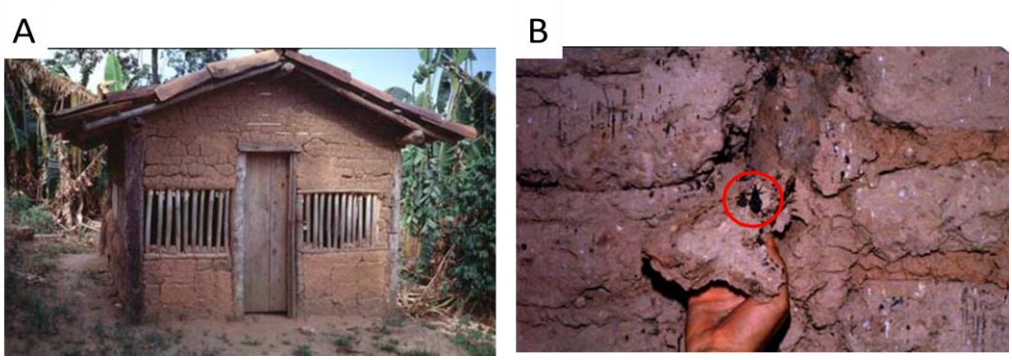


Figure 9 Poorly constructed dwellings as a habitat for triatomine bugs. [A] Example of poor-quality housing construction. [B] Cracked mud walls providing refuge for triatomine bugs (circled). (WHO/TDR/Mark Edwards)

2.2.6 Other routes of infection

Oral infection. Infected insects, crushed during beverage preparation, have been the main source of *T. cruzi* leading to outbreaks of orally-acquired infection (Shikanai-Yasuda & Carvalho, 2011). These have often occurred in periurban and rural areas of non-endemicity. Recent instances include contamination of açai juice, the fruit of the palm *Euterpe oleracea* (Nobrega *et al.*, 2009), and contamination of a guava drink, affecting pupils and personnel at a school in suburban Caracas (Noya *et al*., 2010; Segovia *et al*., 2013). Murine models of oral-route infection have indicated parasite persistence in gastric mucosal epithelium, with a key role for parasite surface glycoproteins gp82 in initiating cell invasion (Yoshida, 2008).

Congenital transmission. A report by PAHO gives a figure of over 14,000 annual cases of congenital Chagas disease in the Americas (PAHO, 2006). In order to prevent acute neonatal infection progressing to chronic, ante-natal serological screening of 'at risk' pregnant women, detection of living parasites in neonates in umbilical cord or venous blood, and serology of infants over 9 months old, in conjunction with benznidazole and nifurtimox therapy, are recommended (Carlier *et al*., 2011).

Transfusional and organ donation. Donation of blood or organs from infected individuals is also a route of *T. cruzi* infection. Screening of donated blood has formed part

of the Chagas disease control programmes (SECTION 1.5), and organ donation has been recognised as a route of infection in endemic and non-endemic settings (SECTION 1.4.3)

2.3 Chagas disease

2.3.1 Clinical manifestations

Acute Phase. Cases of the acute phase last 6-8 weeks, and are seen mainly in children, beginning with the potential development of a nodular, erythematous inflammatory response (inoculation chagoma) 1-3 weeks after the bite of the triatomine. Entry of the infective metacyclic trypomastigote form of the parasite passed in the feeding bug's faeces into the circulation occurs through the wound, abraded skin, or via the conjunctiva. In the latter case, Romaña's sign (conjunctivitis, unilateral palpebral oedema, and preauricular satellite adenopathy) may occur (Prata, 2001) (Figure 10A).

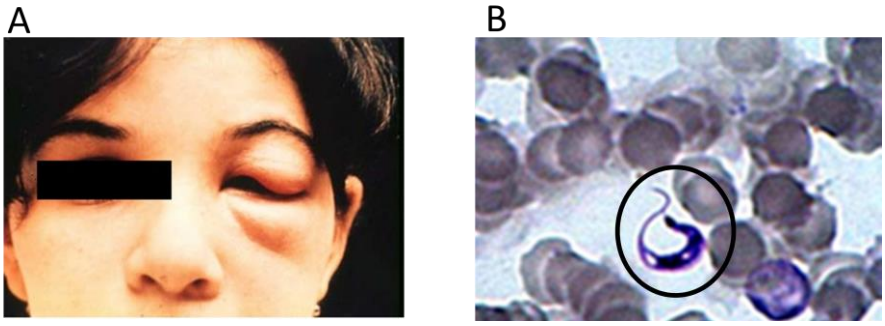


Figure 10 Acute phase of Chagas disease. [A] Romañas sign exhibited in left eye of child. [B] Blood film showing tryptomastigote (circled) with flagellum. (WHO/TDR/Stammers)

Parasitaemia may be identified by direct examination of blood (Figure 10B). The majority of acute cases, however, are not recognised as such, due to the non-specific nature of symptoms (fever, fatigue, oedema), and paucity of clinical manifestations. Without treatment, around 5% of symptomatic patients die during the acute phase due to severe cardiac failure, encephalomyelitis and rarely sudden death (Prata, 2001).

Chronic phase. In the 2-4 months following initial infection, acute symptoms recede, with parasites rarely detected in peripheral blood. The host immune response against the parasite is not sterilizing, *i.e*., does not destroy the first infection or subsequent challenge infections, and the host remains infected for life. The infection may remain clinically latent for decades, and most of these 'indeterminate' patients remain asymptomatic for life. Approximately 30% of infected patients develop the severe form of Chagas disease, in which irreversible chronic manifestations involve the heart, gastro-intestinal tract, and nervous system. Trypomastigote-infected heart muscle cells form amastigote pseudocysts (Figure 11A). When these rupture in the myocardium, an acute myocarditis occurs, with myocytolysis, vasculitis and fibrosis. A high frequency of arrhythmias is characteristic of chronic chagasic heart disease caused by the inflammatory reaction in cardiac tissue. Thinning and deficit is preferentially localised in the apex of the left ventricle (apical lesion; Figure 11B), and is particularly seen in the Southern Cone region of South America. The frequency of this lesion is related to severity of cardiomyopathy, and is accompanied by characteristic electrocardiographic changes. Sudden death occurs in over 30% of chronic cases, mainly in males around the age of 40 (Prata, 2001). Another feature is the presence of chagasic megasyndromes in the southern part of South America. Up to 20% of patients develop functional defects in the oesophagus and colon, due to the destruction of the myoenteric nervous plexus (Dutra & Gollob, 2008). These are marked by the presence of chagasic megaoesophagus (Figure 11C) and megacolon. Dysperistalsis in the oesophagus leads to dysphagia, the most important symptom of oesophagopathy, and megacolonic syndrome often presents as obstipation. Chagas disease may be reactivated by immunosuppression, as occurs in HIV-AIDS, or during organ transplant.

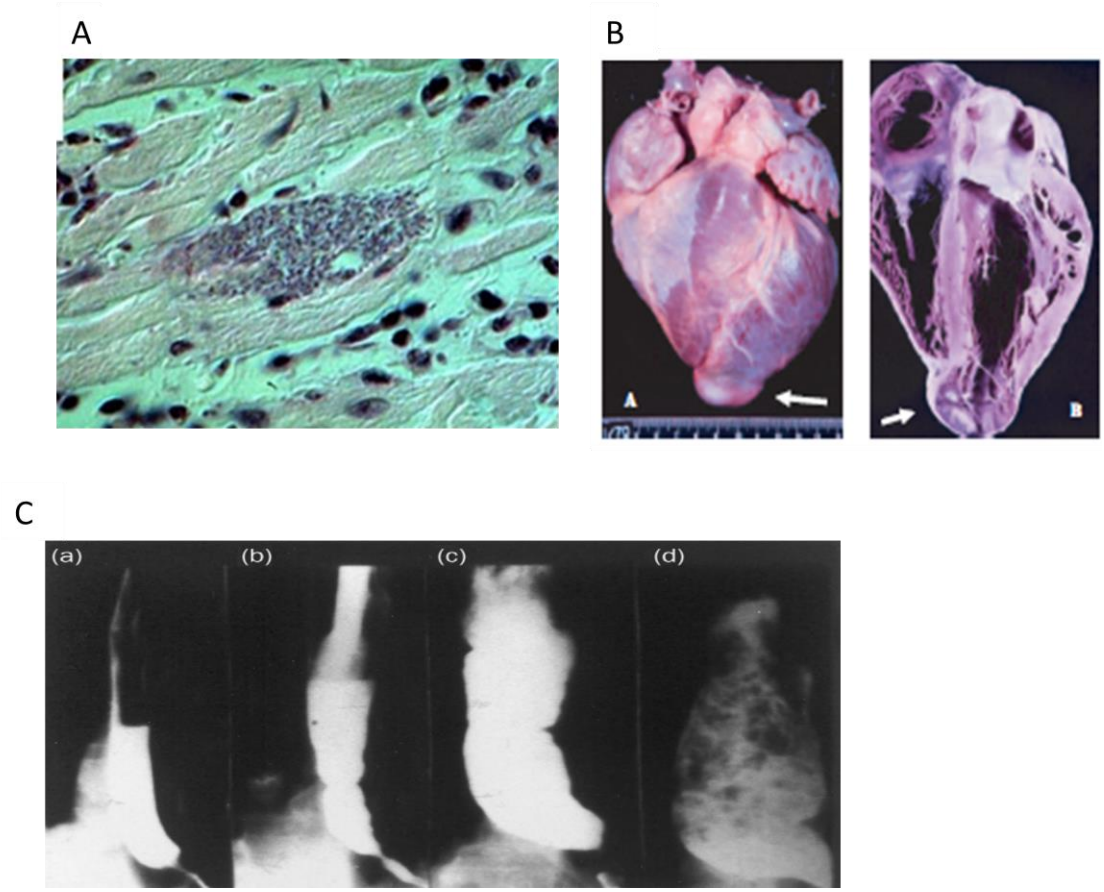


Figure 11 Chronic phase of Chagas disease. [A] Section of heart muscle (haemotoxylin & eosin stain) showing amastigotes. (WHO/TDR/Stammers) [B] (Left) Chagasic heart disease with apical aneurysm (arrowed); (Right) frontal section of same heart showing mild chamber enlargement. (Marin-Neto *et al.*, 2007) [C] Stages of megaoesophagus: (a) anecstatic, (b) mild, (c) moderate, (d) dolichomegaoesophagus. (Prata, 2001)

The course of Chagas disease is represented schematically in Figure 12.

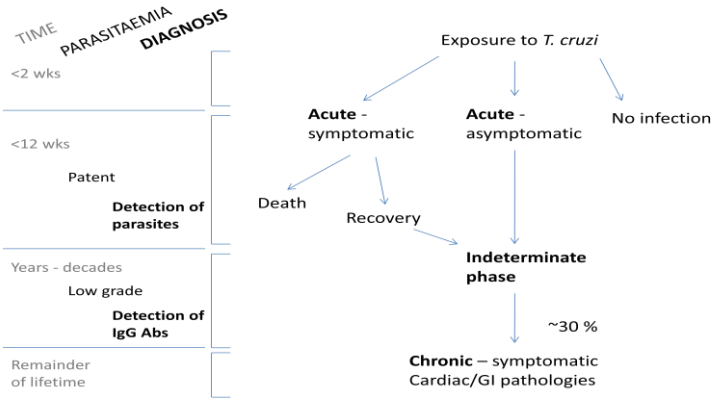


Figure 12 Course of Chagas disease. (Rassi Jr *et al*., 2009)

Parasite load, strain and tissue tropism, and host antiparasite response, autoreactivity and genetic background are thought to be implicated in the establishment of pathology in Chagas disease, and to explain the range of severity of chronic symptoms (Dutra & Gollob, 2008).

2.3.2 Diagnosis

Many of the generalised signs of infection may not lead to identification of acute Chagas disease. *T. cruzi* may be detected parasitologically in the blood in acute cases, but then the parasitaemia decreases to sub-detectable levels. In the chronic phase, xenodiagnosis - the feeding of laboratory-reared triatomines on patient blood and examination for presence of infection in the bug faeces - and blood culture have been used. In this phase, the serological tests used are listed in Table 1 (Anon., 2008).

Table 1 Serological tests used in diagnosis in chronic phase of Chagas disease.

TEST	BASIS	ADVANTAGES	DISADVANTAGES
ELISA	Complex antigen mix	High sensitivity, specificity and throughput; objective quantitative results	Requires a cold chain, technician, and expensive consumable reagents
IIF	Entire parasite	High sensitivity; quantitative and qualitative results	Requires expensive fluorescence microscope and technician; low processing speed.
IHA	Complex antigen mix	Results in ~2 hours; lack of sophisticated equipment; quantitative results.	Lower sensitivity than IFA or ELISA; false positives and low reproducibility; requires cold chain

Current WHO recommendations are that at least two of these tests be positive for definitive serological diagnosis (WHO, 2002). Serological tests use whole cell *T. cruzi* lysates, semi-purified or recombinant antigens for the detection of anti-*T. cruzi* antibodies, which are of the IgG isotype in the chronic phase. A recent WHO comparative evaluation undertaken in Latin America on a panel of commercial serological assays (Otani *et al*., 2009; WHO, 2010d) found that EIA performed better than haemagglutination and particle agglutination assays, with the confirmatory use of RIPA being used as the gold standard for EIA confirmation.

Rapid diagnostic tets (RDTs) generally take the form of immunochromatographic tests, containing whole cell lysates or recombinant antigens bound onto a membrane at a 'test' line, plus a dye-conjugated antibody-binding protein. An example is shown in Figure 13, for the Chagas Stat-Pak Assay RDT. After the sample (whole blood or plasma/serum, depending on manufacturer instructions) is applied in its well, the addition of a larger volume of buffer allows antibodies in the sample to be bound by the dye-conjugated antibody-binding protein, and migrate by lateral flow through the membrane. If the sample contains antibodies against the bound *T. cruzi* antigen, they become localised and can be visualised as a discrete, coloured band on the 'test' line (shown on the right in Figure 13). The sample continues to migrate along the membrane, where an internal IgG antigen 'control' line is visualised, validating the test in the presence or absence of the 'test' line visualisation.

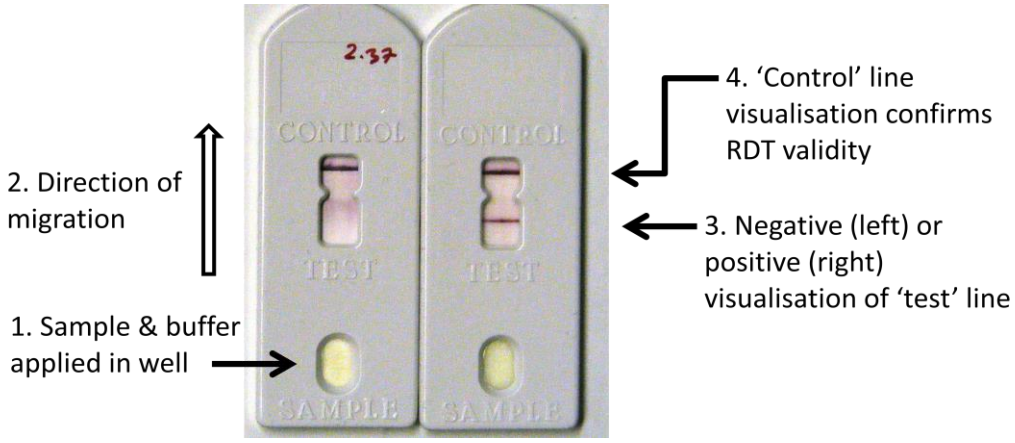


Figure 13 Example of RDT for Chagas disease. In this Chagas StatPak Assay are shown the outcomes if the sample is negative (left) or positive (right) for the presence of anti *T. cruzi* antibodies. Both samples have bands on the 'control' line, thereby validating the test reagents and results.

In a recent international multi-centre trial organised by MSF, a panel of 11 commercialised RDTs was evaluated by National Reference Laboratories in 9 countries (endemic and non-endemic) using their own serum bank plus a standard control panel (Sanchez *et al*., 2014). Although overall performance results were reported as being lower than those described by manufacturers, six tests were recommended by the authors for screening and surveillance as RDTs (with reference laboratory confirmation). Interestingly, one of these was Chagas Stat Pak Assay, which was tested but not recommended by the authors of the WHO Latin American study described above for screening donated blood. The authors of the MSF RDT study also noted that antigenic variability in different geographical regions may play a role in RDT performance; Verani *et al*. (2009) report differences in sensitivities in two RDTs between Bolivian and Peruvian samples.

Diagnosis of cure is assessed by seroconversion (disappearance of anti-*T. cruzi* antibodies); parasite persistence is the sign of therapeutic failure, although this may be difficult to demonstrate. However, after treatment of chronic Chagas disease serological markers may take many years to disappear, so positive serology is not necessarily indicative of active infection. Lack of a proper point-of-care test to assess cure following therapy has been listed as a knowledge gap and research priority in a recent WHO report (WHO, 2012) requiring biomarker discovery and clinical validation.

Another consideration is the possible serological mis-identification of *Trypanosoma rangeli*, a closely-related but non-pathogenic organism, as a false-positive for *T. cruzi* infection (de Moraes *et al.*, 2008), particularly when whole cell lysate is used as antigen. A recent proteomic comparison of a *T. rangeli* strain and a *T. cruzi* strain identified some candidate *T. rangeli*-specific proteins (Wagner *et al*., 2013). The genome of a reference *T. rangeli* strain has recently been published (Stoco *et al*., 2014).

2.3.3 Prevalence

Prevalence of *T. cruzi* infection in Latin America is indicated by country in Figure 14.

Figure 14 Prevalence of *T. cruzi* infection in Latin America.
(www.nature.com/nature/journal/v465/n7301_supp/full/nature09222.html)

Cases of Chagas disease has been identified in non-endemic regions in Europe, North America and Japan in Latin American emigrants (Gascon *et al*., 2009). The number of *T.*

cruzi-infected individuals living in the USA has been estimated at 300,000 (Bern & Montgomery, 2009). Screening of donor blood for *T. cruzi* in the USA has begun recently (CDC, 2007). A WHO report on Chagas disease in Europe describes Spain as having the highest numbers of estimated cases (Figure 15; WHO, 2010c), including most of the congenital infections in babies born to Latin American mothers. Organ transplant from infected individuals has led to reports of Chagas disease in recipients, with recent cases in Spain (umbilical cord blood transplantation; Forés *et al*., 2007) and the USA (heart transplantation; Kun *et al*., 2009).

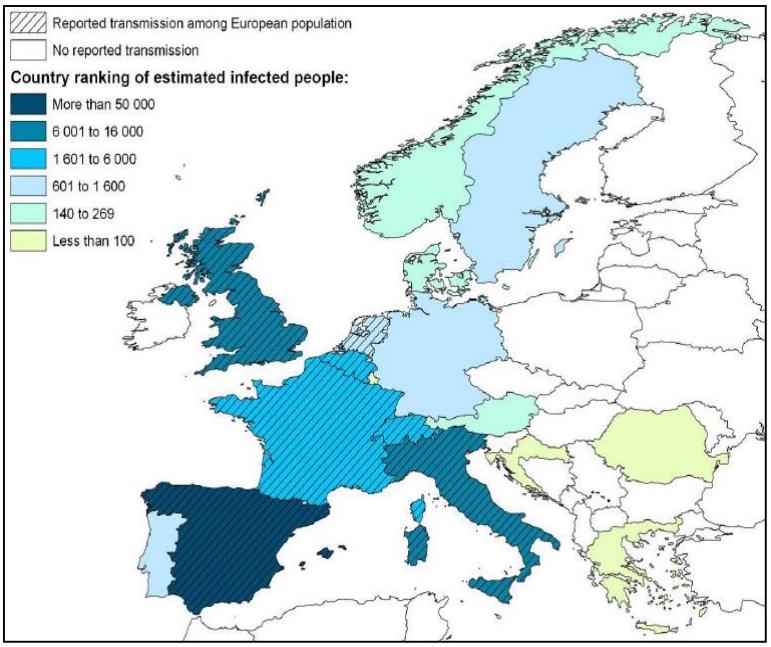


Figure 15 Distribution of cases of *T. cruzi* infection in Europe. (WHO, 2010c).

In the UK, there have been 44 passively diagnosed cases (D Nolder, LSHTM, Personal communication). A figure of 14000 cases of *T. cruzi* infection among Latin American migrants in the UK was recently estimated (WHO, 2010c).

2.3.4 Treatment and Management

No prophylactic vaccine currently exists for Chagas disease. The nitroheterocyclic compounds nifurtimox (developed by Bayer, 1967) and benznidazole (developed by Roche,

1972) have historically been the only available effective drugs for trypanocidal chemotherapy (Figure 16).

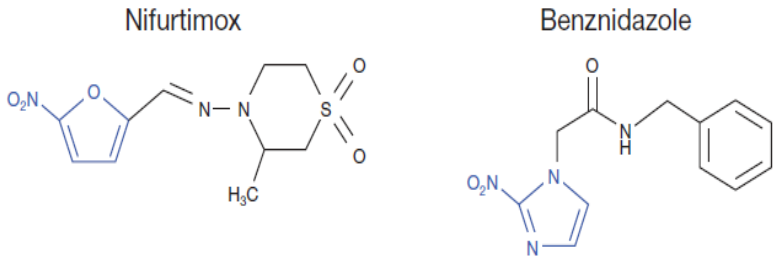


Figure 16 Chemotherapeutic agents to treat Chagas disease. (Wilkinson & Kelly, 2009)

Early treatment by oral chemotherapy during the acute phase requires daily doses for 1-4 months (nifurtimox), and 1-2 months (benznidazole), and can cause severe side-effects. In the case of nifurtimox, these are commonly manifested as gastrointestinal and neurological complications; for benznidazole, allergic dermatitis and peripheral neuropathy (Marin-Neto *et al*., 2009; Bern, 2011). Proposed modes of action of these drugs may be through an increase in oxidative stress in the parasite, and DNA damage (Wilkinson & Kelly, 2009). Nifurtimox and benznidazole are prodrugs, and rely on cleavage by a *T. cruzi*-encoded type-I nitroreductase (TcNTR) for activation to become trypanocidal agents; one mechanism of drug resistance is caused by loss of this gene or its function (Wilkinson *et al*., 2008; Mejia *et al*., 2012). A multinational clinical trial of benznizdole to determine whether trypanocidal treatment improves prognosis in chronic chagasic heart disease patients (the BENEFIT project) is ongoing (Marin-Neto *et al*., 2009; http://clinicaltrialsgov/ct2/show/NCT00123916). The anti-fungal triazoles posaconazole and ravuconazole, ergosterol biosynthesis inhibitors, have recently been used in clinical trials as novel trypanocides. However, posaconazole did not show efficacy in adult South American patients with chronic Chagas disease residing in Barcelona (Molina *et al*., 2014). Ravuconazole, administered in its prodrug form E1224, in trial in chronic inderminate phase patients in Bolivia (http://clinicaltrials.gov/ct2/show/NCT01489228?), also has not shown promising results.

An important consideration is that drug tests should consider the genetic diversity of *T. cruzi* (Zingales *et al*., 2014), further described in SECTION 2.5.

Surgical treatment for megacolon (Nahas *et al.*, 2009) and megaoesophagus is important in chronic cases.

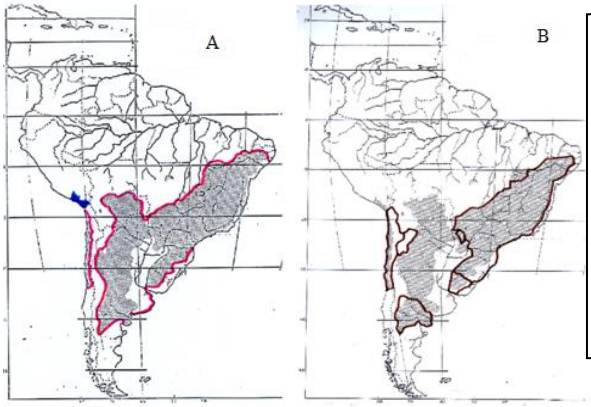
2.4 Control programmes

In recent decades, a number of initiatives have been supported by PAHO with the aim of controlling the spread of *T. cruzi* transmission. These programmes have been directed towards prevention of transmission by transfusion of donated blood, and by residual insecticide spraying of dwellings against the triatomine vectors. These are operated through subregional initiatives, as shown in Figure 17.

Year Initiative	Countries
1991 INCOSUR	Argentina, Bolivia, Brazil, Chile, Paraguay, Uruguay.
1997 IPCA	Belize, Costa Rica, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Panama.
1997 IPA	Colombia, Ecuador, Peru, Venezuela.
2004 AMCHA	Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Peru, Suriname, Venezuela.

Figure 17 Subregional vector control initiatives against *T. cruzi* transmission. (nature.com/nature/journal/v465/n7301_supp/full/nature09222.html); PAHO.

Some key achievements of INCOSUR are described in Figure 18.



1997 Uruguay: Interruption of vectorial and transfusional transmission

1999 Chile: Interruption of vectorial transmission

2000 Interruption of transmission by *T. infestans* throughout most of endemic area

2006 Brazil: formally declared to be free of transmission due to *T. infestans.*

Figure 18 INCOSUR. [A] Areas covered by INCOSUR programme delineated in red. [B] Areas where vectorial transmission by *T. infestans* has been interrupted shown delineated. INSET: some key achievements of INCOSUR. (PAHO; Schofield *et al*., 2006).

In 2008, Guatemala was the first country in Central America to be certified by IPCA as being free from transmission by *R. prolixus*, the main Central American domiciliated vector, followed by Mexico in the same year, El Salvador in 2010, Nicaragua, Honduras and Costa Rica in 2011, and Belize in 2012 (Hashimoto and Schofield, 2012; PAHO, 2012)

2.5 *T. cruzi* ecology and epidemiology

Chagas disease is an anthropozoonosis, in that *T. cruzi* was initially a parasite of sylvatic animals, and became a human infection after triatomine bugs adapted to invading human settlements for a food source. Unlike birds and reptiles, all mammals are considered at risk from *T. cruzi* infection, and are thus potential natural reservoirs. The recognition of a disparate geographic distribution of chagasic disease syndromes, and aspects of *T. cruzi* biology, led in the 1970s to the first description of distinct parasite 'strain-groups' in Brazil, derived from human/domicilated animal infections and from sylvatic opossums and triatomines (Miles *et al*., 1977). Using a MLEE approach, the electrophoretic patterns of various *T. cruzi* enzymes revealed two combinations of isoenzyme pattern among the stocks investigated, leading to the initial subdivision of *T. cruzi* into zymodemes, or enzyme groups. Later molecular (DNA–based) techniques, such as analysis of the spliced-locus intergenic region (SL-IR) of the miniexon gene and 24Sα rDNA (Souto *et al.*, 1996), reinforced the dichotomous view of *T. cruzi*. On the basis of the accumulated data, *T. cruzi* was classified

into two principal groups, TcI and TcII (Anon., 1999). TcII was subsequently further divided into TcIIa-e (Brisse *et al.,* 2000) based on genetic markers. According to the latest recommendations (Zingales *et al*., 2009), the six lineages are now recognised as **TcI - TcVI**. Lewis *et al*. (2011) report the recent, anthropic generation of the TcV and TcVI lineages, resulting from a hybridisation between TcII and TcIII. A scheme for *T. cruzi* lineage evolution is depicted in Figure 19.

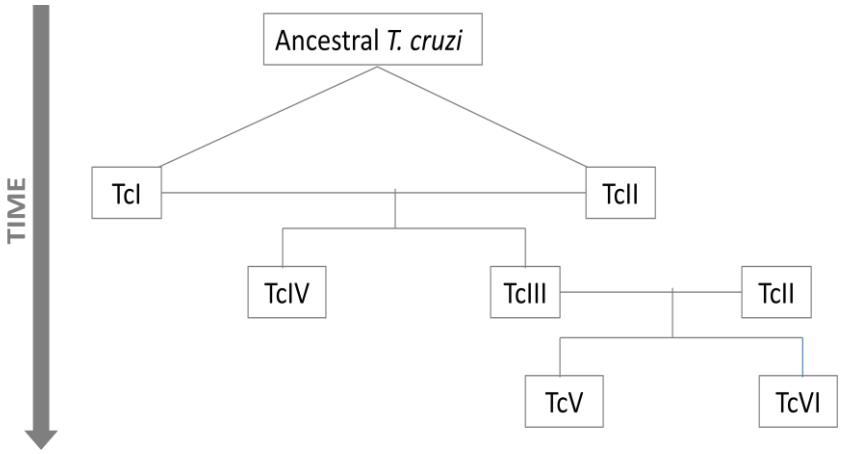


Figure 19 Schematic of proposed *T. cruzi* lineage evolution. (Westenberger *et al*., 2005)

Gaunt *et al.* (2003) used distinct 'parent' strains of *T. cruzi* transfected with different drug resistance markers in co-passage through mammalian cell culture to produce hybrid double-resistant clones *in vitro*, showing that the parasite has an extant capacity for genetic exchange, involving the fusion of parental genotypes, loss of alleles, and homologous recombination.

The association of lineages with geographical distribution is complex (Figure 20). TcI is found as far north as the USA, whereas TcII, TcV, and TcVI predominate in the Southern Cone countries, although TcII infection has been reported in Colombia (Zafra *et al.*, 2008). Recent evidence has shown TcIII to exist in armadillos from Venezuela to Paraguay (Llewellyn *et al*., 2009b), but is rare in humans. Carrasco *et al.* (2012) report around 20% of human-isolated *T. cruzi* in Venezuela to be TcIV. TcI is linked to chagasic cardiomyopathy in central and northern South America, whereas TcII/TcV/TcVI in the Southern Cone correlate in distribution with cardiomyopathy and the presence of chagasic megasyndromes of the

colon and oesophagus. An important goal remains to clarify the relationship between parasite genotype and clinical outcome.

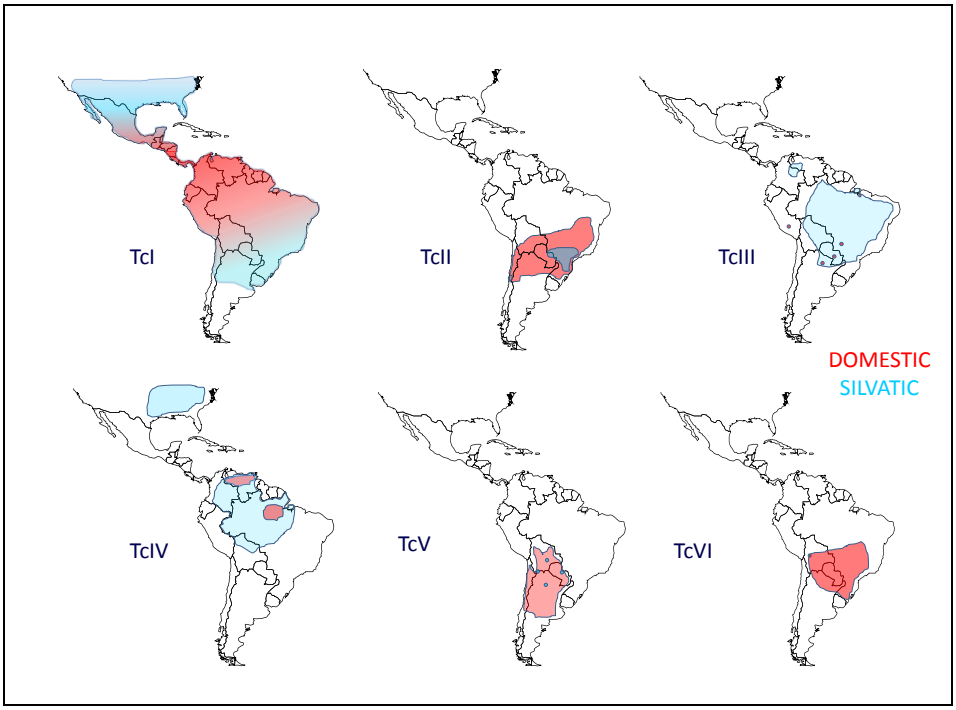


Figure 20 Approximate distributions of *T. cruzi* lineages, with cycles of transmission. (M Llewellyn, LSHTM)

Yeo *et al*. (2005) proposed broadly that TcI co-evolved with opossums (*Didelphis* spp.) or *Rhodnius* in the palm tree niche, whereas, TcIII co-evolved with armadillos (*Dasypus* spp.) in the terrestrial niche (Figure 21). They further suggest that respective historical arboreal and terrestrial ecologies of these hosts explain intraspecific diversity of *T. cruzi* in South America, and the separate origins for Chagas disease in the northern and southern parts of the continent.

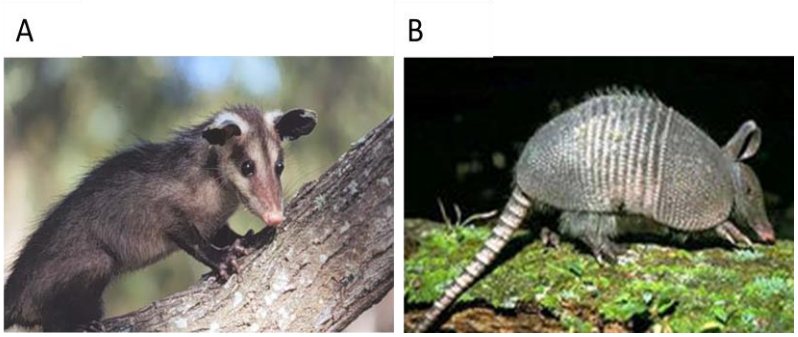


Figure 21 Mammalian hosts of *T. cruzi*. [A] Opossum *Didelphis marsupialis*. (Universidade Federal de Minas Gerais, Brazil). [B] Nine-banded armadillo *Dasypus novemcinctus*. (National Biological Information Infrastructure, USA)

The different lineages are associated with different ecological niches, natural transmission cycles (sylvatic or domestic), and Chagas disease, as summarised in Table 2.

Table 2 Ecological niches and disease associations of *T. cruzi* lineages. (Miles *et al*., 2009)

	Niche	Main sylvatic host	Main sylvatic vectors	Chagas Disease
TcI	Primarily arboreal	*Didelphis* (opossum), other arboreal	*Rhodnius* spp.	North of Amazon: cardiomyopathy.
TcII	Domicilated; uncommonly sylvatic	? Atlantic forest primates	?	Atlantic/Central Brazil. Cardiomyopathy, megasyndromes.
TcIII	Terrestrial/fossorial	*Dasypus novemcinctus* (nine-banded armadillo)	*Panstrongylus geniculatus*	Rarely documented
TcIV	Arboreal primates	Primates, bats, etc	*Rhodnius, Triatoma, Panstrongylus*	Secondary cause in Venezuela; sporadic elsewhere
TcV	Domicilated; rarely sylvatic	? *Dasypus novemcinctus*	?	Southern Cone: cardiomyopathy, megasyndromes.
TcVI	Domicilated; rarely sylvatic	?	?	Southern Cone: cardiomyopathy, megasyndromes.

Lewis *et al*. (2009) used PCR-AFLP and -RFLP protocols to develop a system for lineage identification (Figure 22).

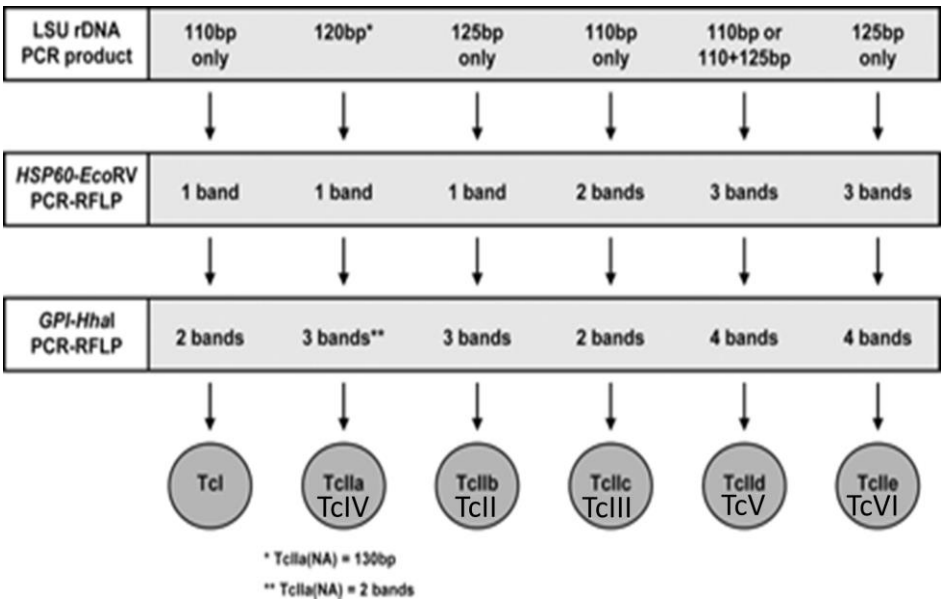


Figure 22 Triple assay for discriminating *T. cruzi* lineages. (Lewis *et al*, 2009). LSU rRNA = large subunit ribosomal RNA gene; HSP60 = heat shock protein 60 gene; GPI = glucose-6-phosphate isomerase. Corresponding current and previous lineage designations are shown.

The phylogenetic relationships between lineages have been explored by the use of multilocus sequence typing (MLST) targets. Concatenated sequences from four loci were used to group a panel of *T. cruzi* isolates into clades, as shown in Figure 23 (Yeo *et al*, 2011). By this analysis, two groupings, one comprised by TcII, TcV and TcVI, and the other by TcI, TcIII and TcIV emerged.

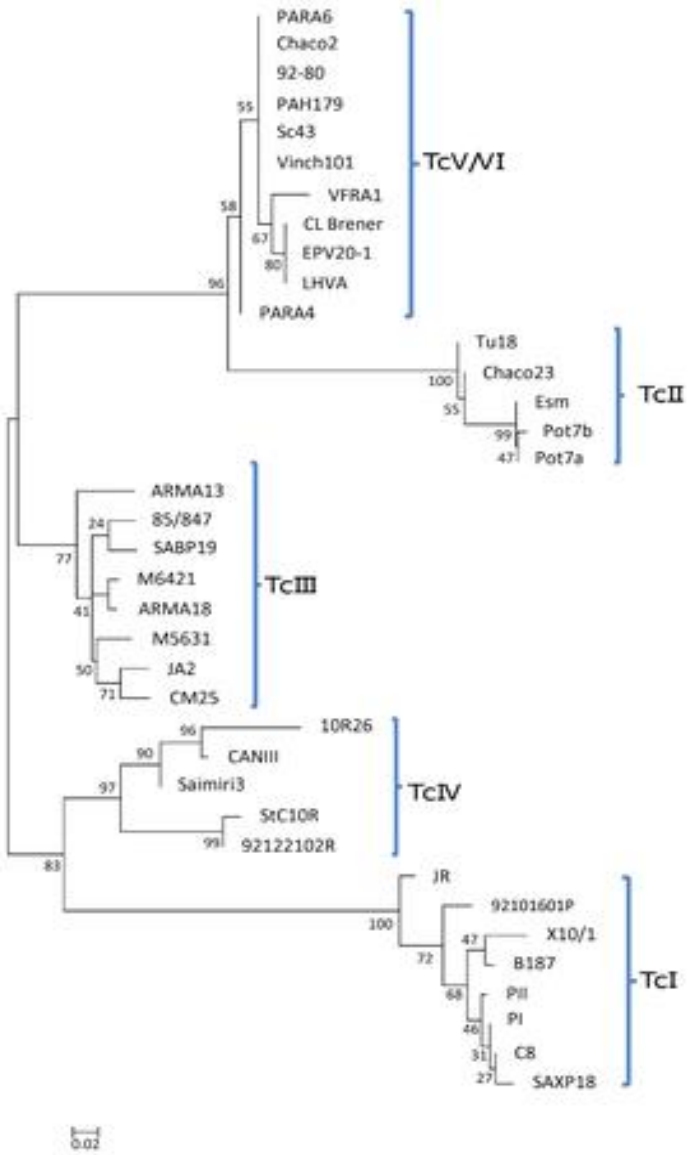


Figure 23 Phylogenetic relationships of T. cruzi lineages. (Yeo *et al*., 2011)

Even within individual lineages, the situation appears more complex than previously understood. Typing based on TcI SL-IR has revealed sub-groups TcIa-e (Falla *et al*., 2009; Cura *et al.,* 2010; Tomasini *et al.,* 2010). Llewellyn *et al*. (2009a) described human isolates restricted to two distinct groups within a broad panel of TcI strains by multilocus microsatellite typing (MLMT) typing. Ocaña-Mayorga *et al*. (2010), described two discrete populations of TcI, one predominantly domestic/peri-domestic, the other silvatic, in Ecuador.

Of interest is the different tissue tropisms of different lineages, which has been reported with analysis of *T. cruzi* DNA from different organs using patient samples taken from heart and oesophagus (Vago *et al*., 2000) or blood and heart (Burgos *et al*., 2010);

recent murine studies have also reported different myocarditis patterns (Rodriguez et al., 2014).

Recently, a new genotype of *T. cruzi* restricted to bats, called TcBat, related to TcI, has been described from Brazil (Marcilli *et al*., 2009), Panama (Pinto *et al*., 2012) and Colombia (Ramirez *et al*., 2013).

However, of crucial importance are the current limitations of identifying infecting lineage: i) direct genotyping of *T. cruzi* by direct PCR on blood or cultured isolates is only feasible when there is a sufficient circulating parasitaemia, which is confounded by the sequestration of the parasite in host tissues in the chronic phase, possibly in a lineage-dependent manner, and the prospect of lineage selection bias during culture; ii) current serological methods to detect antibodies against *T. cruzi* give no information on infecting lineage(s) and are not designed for that purpose.

2.6 *T. cruzi* reference genome sequencing

Table 3 lists the *T. cruzi* strains representative of their lineages for which reference genomes have been sequenced.

Table 3 Reference genomes of *T. cruzi* lineages.

Lineage	strain	Reference
TcI	Silvio X10/1	Franzén *et al*., 2011
TcI	JR4 cl1	Unpublished [a]
TcI	Dm28c	Grisard *et al*., 2014
TcII	Esmeraldo cl3	Unpublished [a]
TcVI	CL Brener	El-Sayed *et al*., 2005
T. c. marinkellei[b]	B7 cl 11	Franzen *et al*., 2012

[a] accessible via http://tritrypdb.org; [b] bat-associated subspecies

As it had been well characterised experimentally, CL Brener (TcVI) was chosen as the strain for the first *T. cruzi* genome sequence (El-Sayed *et al*., 2005). As shown in Figure 19, TcV and TcVI are now understood to be hybrids of TcII and TcIII; thus for the CL Brener genome the two contributing haplotypes were named Esmeraldo-like (TcII) and non-Esmeraldo-like (TcIII). Additionally, over 50% of this genome was found to consist of repeated sequences. The genome of a representative TcI strain, Sylvio X10/1, was subsequently sequenced as a non-hybrid, evolutionarily distinct, comparison (Franzén *et al*., 2011). The TcI Sylvio X10/1 haploid genome was estimated to be smaller than that for TcVI CL Brener (44 Mb *vs*. 55 Mb). The two genomes were reported to have highly conserved gene synteny and core gene complement, but although 6 ORFs in CL Brener were not found in Sylvio X10/1, no Sylvio X10/1-specific genes were identified. The comparative genomic analysis also found a differential expansion in sequences corresponding to surface antigen repertoires and other multicopy gene families, with the CL Brener genome estimated as having ~6Mb of extra haploid sequence related to these multigene families compared to Sylvio X10/1. As surface molecule genes are major contributors to the repetitive nature of the CL Brener genome, this was concluded by the authors to underlie most of the haploid genome size difference between the two *T. cruzi* lineages, having the potential to enhance functional plasticity.

2.7 Research needs: Chagas disease

As described herein, *Trypanosoma cruzi* is now understood to be a complex of diverse genotypes, possessing the capacity for genetic exchange, and displaying differing geographical distributions in terms of Chagas disease symptoms and severity, sylvatic hosts, and transmission cycles. Control programmes underway in recent years have the potential to achieve even greater successes in interrupting transfusional and vector-borne transmission. Relevant to this project, Coura (2007) lists a 'top ten of Chagas disease needs' including '3. To improve a specific and fast test for TcI-TcVI infections' and '4. To standardize the techniques for serological diagnosis and control of cure evaluation'.

Thus, there is the key question of the identification of an individual's history of *T. cruzi* lineage infection. As described above, molecular (DNA) techniques have their

limitations, so an indirect approach is required. Such a method would be based on the identification of lineage-specific antigens and the development of a serological assay to exploit differential antibody reponses to these.

Aims and objectives for the work on *T. cruzi* presented in this thesis are described in SECTION 3.1.

2.8 Discovery of *Leishmania*

In 1903, William Leishman (1865-1926) and Charles Donovan (1863-1951) both reported observations of intracellular bodies in samples from 'kala-azar' patients in India (Figure 24; Leishman, 1903; Donovan, 1903). In the same year, Ronald Ross (1857-1932) gave the name *Leishmania donovani* to these intracellular parasite stages (Ross, 1903). Person-to-person transmission was later demonstrated to be mediated by the sandfly (Swaminath *et al*., 1942).

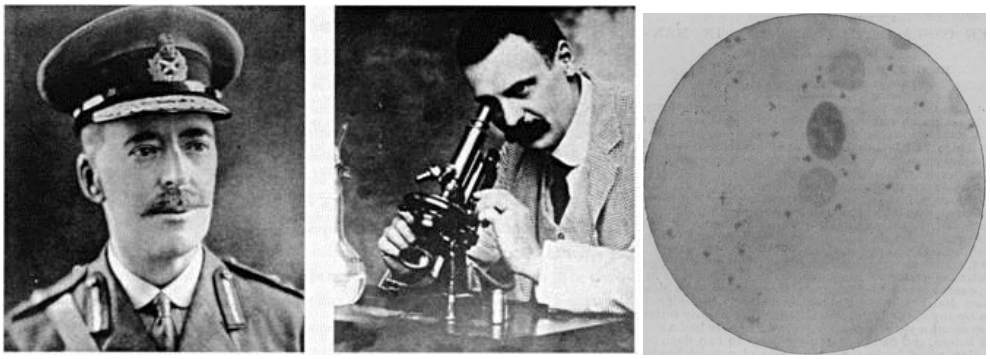


Figure 24 Leishman and Donovan. [Left] William Leishman. [Centre] Charles Donovan. (both http://dna.kdna.ucla.edu/parasite_course-old/leish_files/subchapters/Historical.htm). [Right] Spleen smear preparation from patient described in Leishman (1903).

2.9 *Leishmania* spp.

2.9.1 Taxonomy

The taxonomy of *Leishmania* to genus level is shown in Figure 4. *Leishmania* are classified into the two subgenera *Leishmania* (*Leishmania*), and *Leishmania* (*Viannia*). Taxonomy to species level is shown in Figure 25.

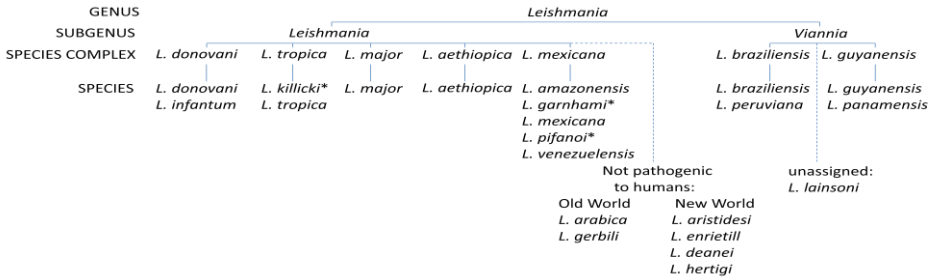


Figure 25 Taxonomy of *Leishmania*. * = species status under discussion. (WHO, 2010b)

2.9.2 Life cycle

The life cycle of *Leishmania* is summarised in Figure 26.

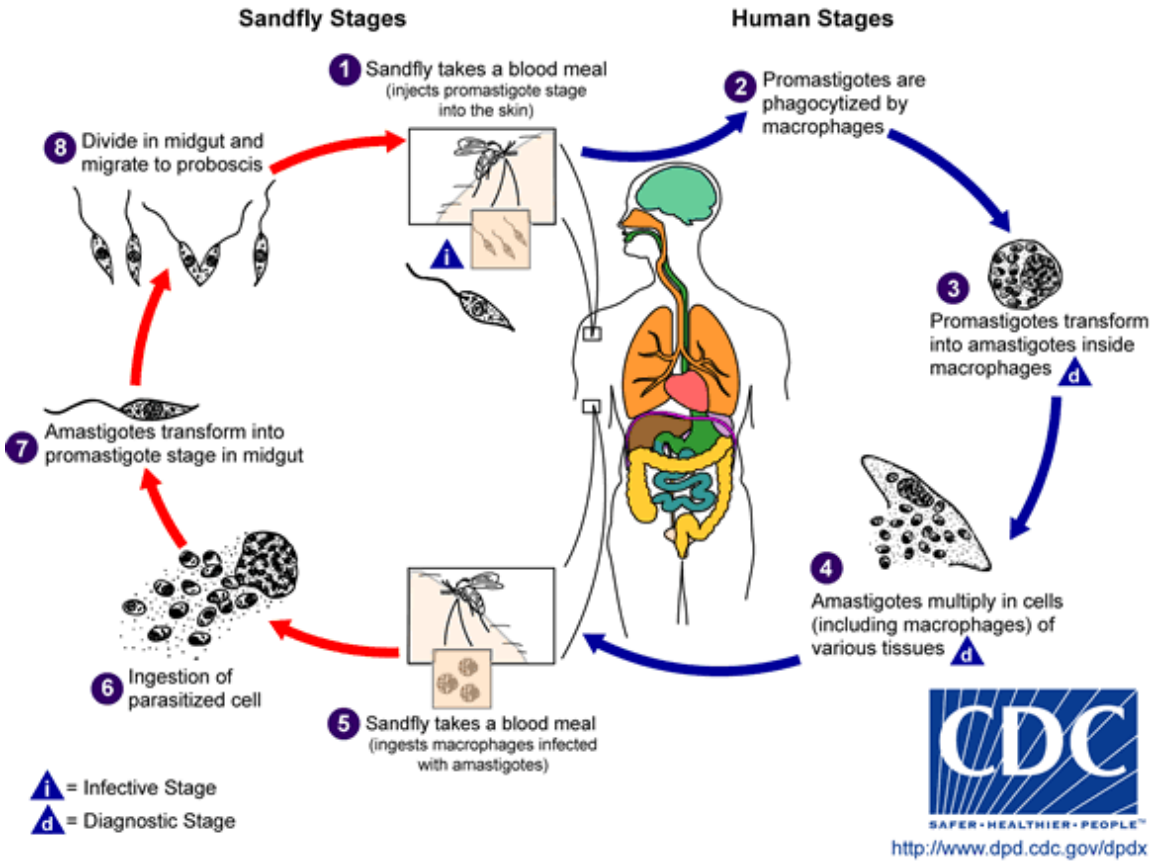


Figure 26 *Leishmania* life cycle. (CDC)

Leishmania promastigotes are transmitted to humans through the proboscis of female sandflies (*Phlebotomus* spp. and *Lutzomyia* spp. in the Old and New World respectively) during bloodmeal feeding. Following internalisation by local dermal macrophages and dendritic cells, flagella are lost, and transformation into proliferative amastigote forms leads to cell lysis, and phagocytosis by other cells. Upon taking a bloodmeal containing infected host macrophages, amastigotes are liberated in the sand fly gut, transform into promastigotes, and migrate to the proboscis (described in more detail in SECTION 2.9.3).

2.9.3 Vectors

The vectors for *L. donovani* are *Phlebotomus argentipes* in South Asia, *Phlebotomus martini* and *Phlebotomus orientalis* in East Africa, and *Lutzomyia longipalpis* for *L. infantum* in the Americas (Figure 27).

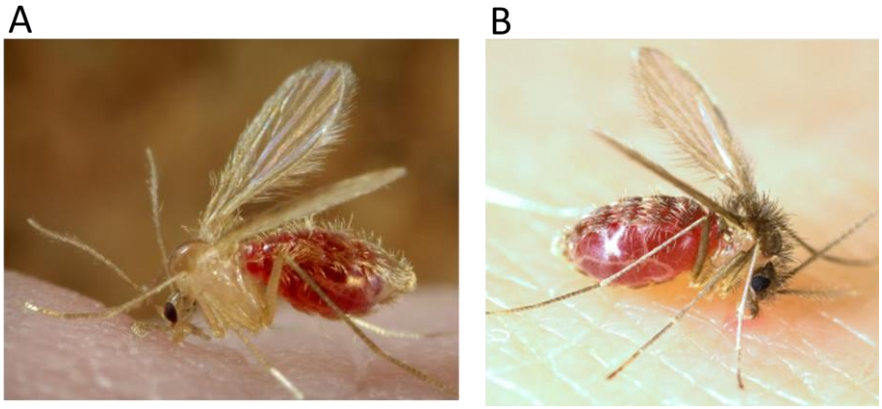


Figure 27 Sandfly vectors of *Leishmania*. [A] *Phlebotomus* sp. (Old World vector). (CDC) [B] *Lutzomyia longipalpis* (New World vector). (www.plospathogens.org/article/fetchObject.action?uri=info%3Adoi%2F10.1371%2Fimage.ppat.v05.i08.g001&representation=PNG_S)

Leishmania are classified into the two subgenera *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*). In *Leishmania* (*Leishmania*), promastigote development occurs in the sandfly midgut (suprapylarian development), whereas it occurs in the hindgut (peripylarian development) for *Leishmania* (*Viannia*). Species of the subgenus *L.* (*Viannia*) are restricted to the New World. Suprapylarian development, the better understood of the two, is briefly described here (Kamhawi, 2006; Bates, 2007). *Leishmania* amastigotes ingested by the anthropophilic sandfly during bloodmeal feeding transform first into procyclic promastigotes in the blood bolus, then into motile nectomanad promastigotes (Figure 28). These migrate along the midgut epithelium by binding of surface lipophosphoglyan to a receptor in the sandfly gut. Subsequent transformation into replicative leptomonad promastigotes in the anterior midgut precedes differentiation into non-replicative infectious metacyclic promastigotes and also haptomonad promastigotes, which attach to and plug the opening of the stomodeal valve. Promastigote secretory gel produced by leptomanads fills the thoracic midgut limen; the resulting 'blocked fly' effect enhances regurgitation of metacyclic promastigotes into the host during bloodmeal feeding (Rogers *et al*., 2004).

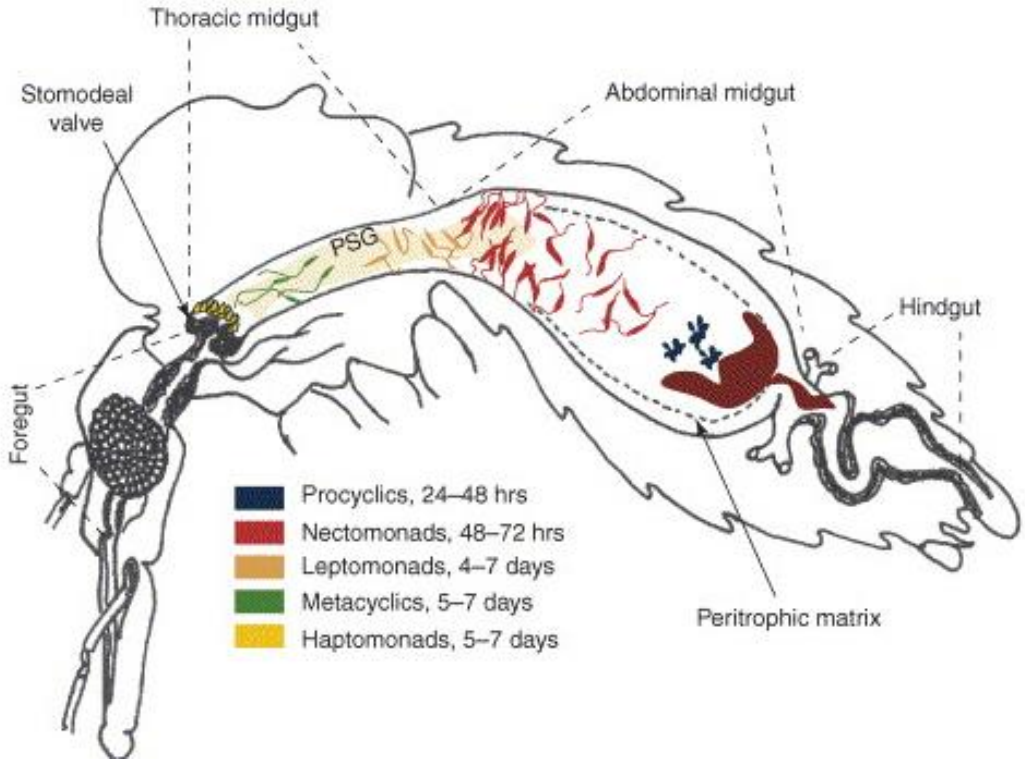


Figure 28 *Leishmania* development in the sandfly vector (suprapylarian). PSG = Promastigote secretory gel. (Kamhawi, 2006)

2.10 Visceral leishmaniasis

2.10.1 Clinical manifestations

Following infection from a female sandfly, the incubation period can range from 10 days to over 1 year, and usually disease onset is gradual. Common symptoms are fever, malaise, shivering/chills, weight loss, anorexia and discomfort in the left hypochondrium. Clinical signs are non-tender splenomegaly, sometimes with hepatomegaly, wasting and pallor of mucous membranes (Figure 29A). Lymphadenopathy may be present (particularly in Sudan) and be the only clinical manifestation. Darkening of the skin of the face, abdomen, hands, and feet may be found in India (Hindi: kala-azar = black fever). In Sudan, a *Leishmania*-containing cutaneous nodule or ulcer or a mucosal lesion may be present. Signs of malnutrition (oedema, skin and hair changes) develop as the disease progresses. In advanced stages, intercurrent infections by other pathogens are common (WHO, 2010b). Post-Kala Azar Dermal Leishmaniasis (PKDL), characterized by a hypopigmented macular,

maculopapular, and nodular rash, may occur in patients who are cured of VL and otherwise well (Figure 29B).

Figure 29 Clinical manifestations of VL. [A] Splenomegaly. (www.sciencephoto.com) [B] PKDL (www.who.int/leishmaniasis/Viscerial_Leishmaniasis_hr.jpg)

PKDL is found in both East Africa and South Asia, with differing epidemiological features, as listed in Table 4 (Zijlstra *et al*., 2003).

Table 4 Epidemiological features of PKDL in Sudan and India. (Zijlstra *et al*., 2003)

	Sudan	India
Frequency following treated VL	50 %	5-10%
Most common interval after VL	0-6 months	2-3 years
Age & sex distribution	Children (mean age 6 yrs), equally male and female	Young adults, male predominance

PKDL may have role in maintaining a reservoir population of *L. donovani*, contributing to disease persistence in the community. The inter-relationship between manifestations of *Leishmania* infection in Sudan is shown in Figure 30 (Zijlstra *et al*., 2003).

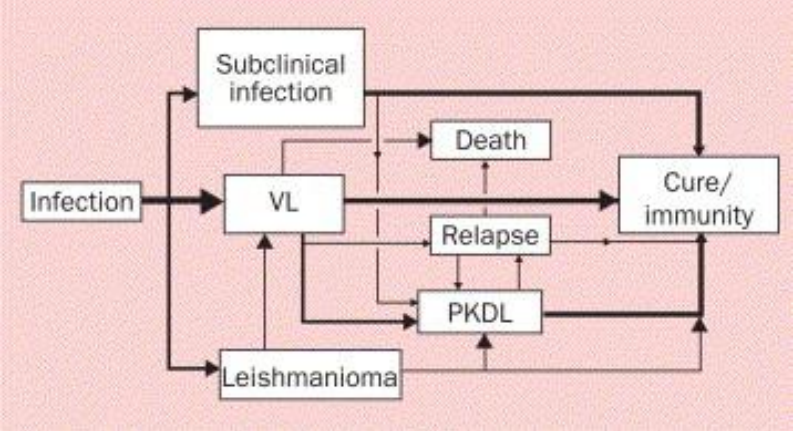


Figure 30 Inter-relationship between manifestations of *Leishmania* infection in Sudan. Line thickness corresponds with likelihood of occurrence. (From Zijlstra *et al.*, 2003)

Elevated levels of the immunosuppressive cytokine interleukin-10 (IL-10) in the serum, and elevated IL-10 mRNA in spleen or bone marrow, have been often reported in VL (Nylen and Sacks, 2007); IL-10–neutralising monoclonal antibodies promoted amastigote clearance *ex vivo* in splenic aspirate cells from VL patients (Gautum *et al*., 2011). There is a mixed Th1/Th2 response in human VL (WHO, 2010b).

2.10.2 Diagnosis

Clinically suspected cases are those exhibiting fever for more than 2 weeks and splenomegaly in the absence of malaria (WHO, 2010b). Parasitological diagnosis, by demonstration of amastigote in lymph node, spleen, or bone marrow tissue smear, is the definitive diagnosis (Figure 31).

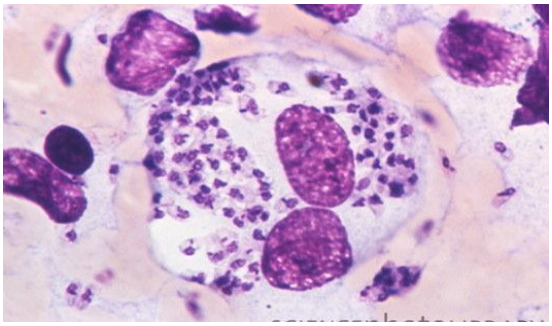


Figure 31 *L. donovani* amastigotes inside infected macrophage. Light micrograph of spleen tissue section. (www.sciencephoto.com).

Serological (anti-*Leishmania* antibody) tests include ELISA, IFAT, and DAT (Srivastra *et al*., 2011). For the last few years, the RDT for VL widely used has been the rK39 ICT, based on a kinesin-related gene product, LcKin, identified by screening a Brazilian *L. infantum* (*L. chagasi*) genomic library with serum of an *L. donovani* patient (Burns *et al*., 1993). A part of the coding sequence, comprising a 46 aa region followed by 6.5 x 39 aa repeats, forms the recombinant diagnostic protein rK39, used in the RDT bound on an ICT strip. The presence of anti-*Leishmania* antibodies in the patient sample can be visualised in a few minutes after application by the presence of a coloured band on the test strip, in the same manner as described in Section 2.3.2. An example for the rK39 RDT (BioRad Laboratories) used with Sudanese VL sera is shown in Figure 32.

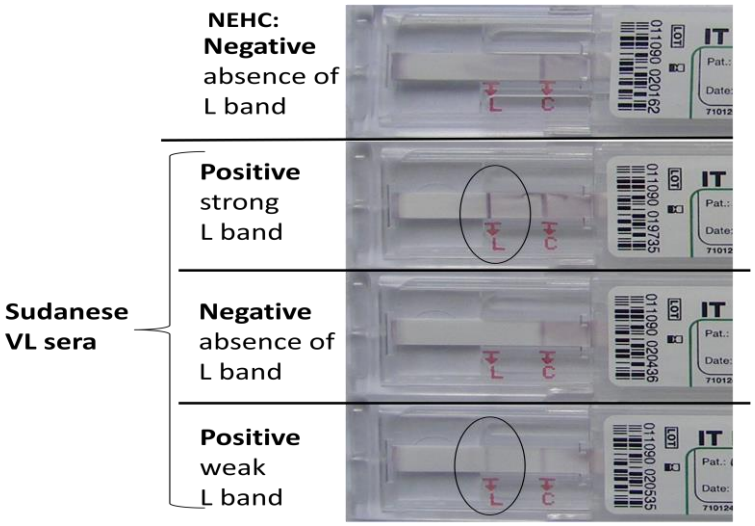


Figure 32 rK39 RDT for VL. Sudanese sera were applied to the RDT. The presence of a band at the L line indicates the presence of anti-*Leishmania* IgG. NEHC = non-endemic healthy control.

In recent multicentre evaluations, the use of the rK39 ICT RDT reported less success in East Africa than in the Indian subcontinent for point-of-care diagnosis of VL (Tables 5 & 6; Boelaert *et al*., 2008; Cunningham *et al*., 2012).

Table 5 Boelaert *et al*., 2008: Prevalence in the test sample and sensitivity and specificity of rK39 dipstick (with 95% CI).

	East Africa			South Asia	
	Ethiopia	Kenya	Sudan	India	Nepal
Prevalence	57.2 (40.5-73.4)	60.9 (54.7-66.7)	37.0 (31.0-43.2)	79.6 (75.1-83.7)	71.0 (63.5-77.9)
Sensitivity	75.4 (55.9-90.5)	84.7 (78.6-89.8)	77.9 (69.2-85.6)	99.6 (98.4-100)	96.5 (92.1-99.2)
Specificity	70.0 (46.3-88.9)	89.9 (83.2-95.1)	91.8 (86.7-96.2)	90.0 (81.2-96.4)	90.9 (80.8-97.5)

Table 6 Cunningham *et al*., 2012: Sensitivity and specificity of rK39 rapid diagnostic tests (with 95% CI).

		East Africa	Brazil	Indian subcontinent
'DiaMed-IT Leish' (Bio-Rad)	Sensitivity	87.2% (82.5%-90.8%)	92.0% (87.8%–94.8%	98.8% (96.5%–99.6%)
	Specificity	96.4% (93.3%–98.1%)	95.6% (92.2%–97.5%)	97.6% (94.8%–98.9%)
'KalaAzar Detect' (InBios)	Sensitivity	67.6% (61.6%–73.1%)	84.7% (79.7%–88.7%)	99.6% (97.8%–99.9%)
	Specificity	90.8% (86.6%–93.8%)	96.8% (93.9%–98.4%)	96.0% (92.8%–97.8%)

Underlying explanatory factors for this differential regional sensitivity may reside in molecular divergence between East African *L. donovani* kinesin gene homologues and the Brazilian *L. infantum* (*L. chagasi*)-derived rK39 sequence and/or may be due to differential immunocompetence and antibody levels produced among African and Asian human populations.

The first two of the 39 aa repeats of Sudanese *L. donovani* kinesin homologue LdKin (Gerald *et al*., 2007), flanked by sequences of the *L. donovani* antigens HASPB1 and HASPB2 (Alce *et al*., 1999), comprise rK28 (Figure 33), a novel synthetic recombinant protein for diagnosis of VL, designed to be an improvement on rK39 (Pattabhi *et al*., 2010, Vaish *et al*., 2012). HASPB proteins are expressed on the surface of infective promastigotes and amastigote. The first 3 x 14 aa repeats of HASPB1 are incorporated into rK28, along with the complete ORF of HASPB2, which includes three imperfect consecutive repeats, 2 x 14 aa and 1 x 13 aa.

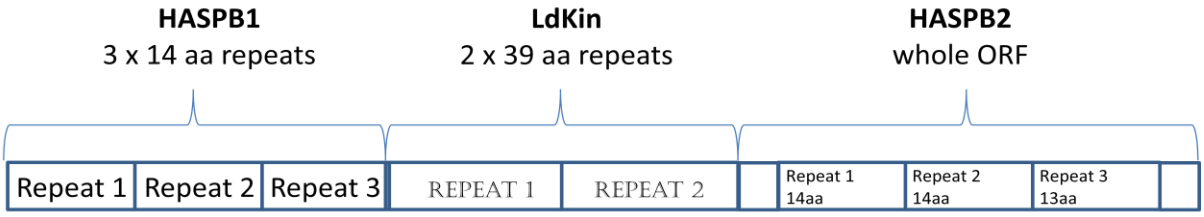


Figure 33 Synthetic gene rK28. Comprised by repeats of HASPB proteins flanking Sudanese-derived kinesin repeats.

An additional aspect of the current serological tests is that they may give positive results post-cure and so cannot readily diagnose relapse, and can also indicate the presence of anti-leishmanial antibodies in asymptomatics living in endemic areas, but with no VL history (WHO, 2010b). A large Indian/Nepalese population study reported an association between higher DAT and/or rK39 titres and risk of progression from asymptomatic to symptomatic VL (Hasker *et al*., 2014); in a long-term follow-up of 55 rK39 seropositive asymptomatic cases in India 69% developed VL and 31% remained asymptomatic (Singh *et al*., 2002). The proportion of seropositive asymptomatic individuals that progresses to symptomatic VL has been reported to vary between endemic regions, as shown in Table 7. Currently there is no rapid diagnostic test that determines or predicts which asymptomatic carriers will progress to active VL.

Table 7 Asymptomatic progression to VL. Reports of the proportions of asymptomatic seropositive individuals who either remain symptom-free or do progress to symptomatic VL are expressed as ratios.

Country	Non progressors: progressors	Reference
Sudan	1:2.4	Zijlstra *et al*., 1994
Kenya	4:1	Schaefer *et al*., 1995
Brazil	8:1	Evans *et al*., 1992
Bangladesh	4:1	Bern *et al*., 2007
India & Nepal	8.9:1	Ostyn *et al*., 2011
Spain	50:1	Moral *et al*., 2002

2.10.3 Treatment and management

Historically, the standard first-line chemotherapeutic agents to treat VL have been the pentavalent antimonials sodium stibogluconate and meglumine antimoniate, administered by injection, although development of resistance to antimonials has emerged in India (Chakravarty & Sundar, 2010), possibly caused by chronic exposure to arsenic in drinking water (Perry *et al*., 2013). More recent anti-leishmanials include amphotericin B, paromomycin, and miltefosine (WHO, 2010b) (Figure 34).

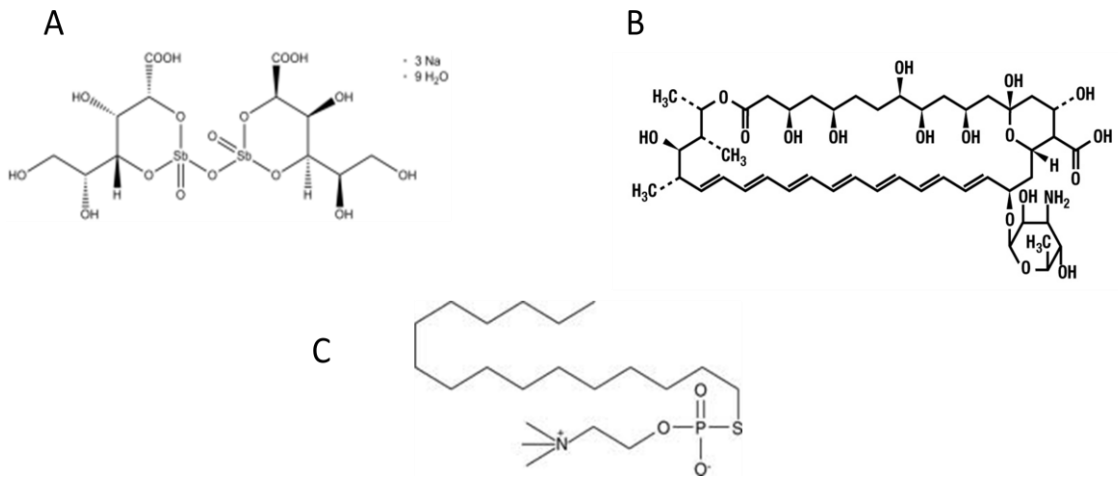


Figure 34 Chemotherapeutic agents to treat VL. [A] sodium stibogluconate. (www.speciation.net) [B] Amphotericin B. (www.ambisome.com) [C] Miltefosine. (www.caymanchem.com)

In South Asia, amphotericin B in liposomal formulation (AmBisome) is the preferred treatment, followed by combination therapies (AmBisome with miltefosine or paromomycicn, or miltefosine with paromomycin) (WHO, 2010b). The primary target for amphotericin B is the surface membrane ergosterol (human cells have cholesterol as their major surface membrane sterol), thus parasite destruction can be achieved without amphotericin B uptake by the host. In East Africa, pentavalent antimonials with paromomycin or in combination or monotherapy remain the preferred treatment choices (WHO, 2010b).

According to a WHO/TDR publication, VL treatment outcomes are assessed twice, an inital outcome on the last day of drug treatment, and a final outcome six months after the last drug was taken (WHO, 2010e). The main outcomes, as listed therein, are given in Table 8.

Table 8 Treatment outcomes in VL (WHO, 2010e).

	Outcome	Case definition
Initial assessment (last day of drug treatment)	Initial cure	A full course of drugs has been completed AND the patient has clinically improved. Clinical criteria for cure should be assessed as no fever + regression of enlarged spleen + return of appetite and/or gain in body weight.
	Non-response	Signs and symptoms persist or recur. Switch to a second-line drug because of no response to the first-line drug.
	Side-effects related switch	Side-effects necessitate a change of treatment.
	Death	Any death, whether or not related to VL.
	Default	The patient does not complete treatment and/or does not present for assessment after treatment.
Final assessment (6 months after last drug taken)	Final cure	An initial cure patient who is symptom-free at six months after the end of treatment.
	Relapse	Any reappearance of KA symptoms within a period of six months after the end of treatment.
	Death	Any death, whether or not related to VL.
	Loss to follow-up	Patient does not present for assessment at six months.

However, Rijal *et al*., (2013), in following up miltefosine–treated patients for up to 12 months after completion of therapy found a relapse rate of 20% (24/120 patients) in their studied cohort in Nepal.

As shown in Table 8, criteria for assessing cure after VL therapy are clinical. The requirement for an established biomarker of cure in VL, its clinical validation and incorporation into a point-of care test have been identified as knowledge gaps and research priorities in a recent WHO report (WHO, 2012).

2.11 Control strategies

In 2005, a regional strategic framework for VL elimination was agreed by the endemic countries of India, Nepal, and Bangladesh, with the target to reduce annual incidence to < 1/10, 000 population at district/sub-district level by 2015 (WHO, 2005). It describes the following factors as favourable for VL elimination: man is the only reservoir host; *P. argentipes* is the only known vector; availability and effectiveness of oral miltefosine; availability of reliable rK39 tests; past effectiveness of indoor residual spraying to control vector; political commitment; focusing of elimination efforts in endemic districts. However, the following constraints are mentioned: incomplete or inappropriate treatment from private doctors/quacks [*sic*]; delay in treatment favouring continued transmission; resistance to antimony drugs; persistence of reservoir in undetected and untreated PKDL; summer outdoor sleeping exposes to peri-domestic vectors; expense of treatment for poorest people.

Indoor residual spraying of organochlorines (*e.g.*, DDT) has been the main strategy to control endophilic sandfly vectors in South Asia (WHO, 2011). Preparedness for, rapid assessment of, and response to, epidemic outbreaks has also described as a component of control strategies (WHO, 2010b).

2.12 *L. donovani* complex population structure

MLEE has been the 'gold standard' for *Leishmania* typing to species level; the Montpelier system (MON) is based on 15 enzymes (Rioux *et al*., 1990). Drawbacks of MLEE typing come from the need to have a large culture of parasite, protein electrophoresis equipment, and the reliance on electrophoretic mobilities of proteins that does not resolve individual amino acid polymorphisms (conserved or non-conserved) caused by coding DNA

changes. More recently, molecular biology techniques based on MLST (Mauricio *et al*., 2006; Zemanova *et al*., 2007) and MLMT have been used to provide greater resolutive power for the *L. donovani* complex. Lukeš *et al*. (2007) showed that the genetic structure of *L. donovani* complex is determined mainly by geography rather than MLEE-based taxonomy or clinical outcome, and proposed an evolutionary hypothesis for the origin and dispersal of *Leishmania*. (Figure 35A,B). The evolution of *Leishmania* was proposed to have taken place in South America ~46-36 mya (Figure 35A). Subsequent dispersal via the Bering land bridge was into Asia, where, 25-15 mya, the ancestor of *L. donovani* complex diverged from other species. Further dispersal led to the emergence of *L. infantum* ~1 mya in Central Asia and moving into Europe, while *L. donovani* entered East Africa and South Asia. *L. infantum* was introduced to the Americas from Iberia by human activities multiple times during the last 500 years; historically this has been called *L. chagasi* (Kuhls *et al*., 2011). When New World *Lutzomyia* became competent vectors for these parasites, VL was thus established in this region. In this thesis and its annexes, the term '*L. infantum* (*L. chagasi*)' is used to describe these parasites.

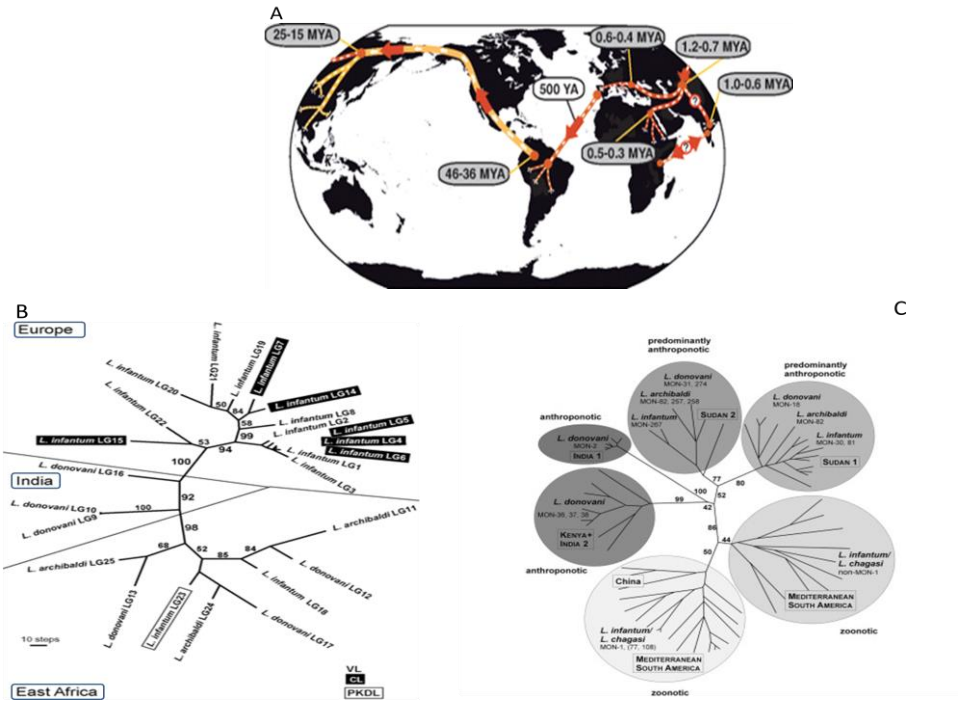


Figure 35 *L. donovani* complex population structure, evolution and dispersal. [A] Proposed model of *Leishmania* evolution and dispersa. MYA = millions of years ago. [B] Genotype is strongly correlated with geographical origin (Lukes *et al*., 2007). [C] MLMT identifies six populations (Kuhls *et al*., 2007).

Kuhls *et al*. (2007) used MLMT to define six genetically distinct populations of *L. donovani* complex in VL endemic regions (Figure 35C), and reported that allelic diversity in Africa, although lower than in the Mediterranean region, was greater than in India. Another MLMT study showed a high degree of homogeneity among South Asian *L. donovani* (Alam *et al*., 2009).

Hybrids and genetic exchange between *Leishmania* of the same species, and between different species, have been described naturally and experimentally (Tables 9 and 10).

Table 9 Natural hybrids of *Leishmania* spp.

Isolated from	Assay	Species	n	Reference
Homo sapiens, Nicaragua	MLEE	*L. panamensis – L. braziliensis*	8	Darce *et al*., 1991
Canis familiaris and *Psammomys obesus*, Saudi Arabia	MLEE, DNA probes	*L. major - L. arabica*	2	Kelly *et al*., 1991
Homo sapiens, NIcaragua	MLEE, DNA probes	*L. panamensis – L. braziliensis*	11	Belli *et al*., 1994
Homo sapiens, Peru	MLEE, RAPD, DNA probes	*L. braziliensis – L. peruviana*	4	Dujardin *et al*., 1995
Homo sapiens, Venezuela	MLEE	*L. braziliensis – L. guyanensis*	4	Delgado *et al*., 1997
Homo sapiens, Ecuador	MLEE, RAPD	*L. braziliensis – L. panamensis/guyanensis*	4	Bañuls *et al*., 1997
Homo sapiens, Portugal	MLST, MLEE	*L. infantum - L. major*	2	Ravel *et al*., 2006
Homo sapiens and *Canis familiaris*, Peru	MLEE, MLMT	*L. braziliensis – L. peruviana*	26	Nolder *et al*., 2007
Homo sapiens and *Canis familiaris*, Tunisia	MLST	*L. infantum- L. infantum*	4	Chargui *et al*., 2009
Homo sapiens, Ethiopia	AFLP	*L. donovani – L. aethiopica*	1	Odiwour *et al*., 2011
Phlebotomus tobbi and *Homo sapiens*, Turkey	Whole genome sequencing	*L. infantum*	a	Rogers *et al*., 2014
Homo sapiens, Ethiopia	MLMT, MLST	*L. donovani - L. donovani*	4	Gelanew *et al*., 2014

[a] Authors report patchy heterozygosity and SNP density across sample set.

Table 10 Experimental hybrids of *Leishmania* spp.

System	Method	Species	Reference
Phlebotomus duboscqi	Drug resistance transfer	*L. major* - *L. major*	Akopyants *et al*., 2009
Phlebotomus perniciosus and *Lutzomyia longipalpis*	Fluorescence gene transfer	*L. donovani* - *L. donovani*	Sadlova *et al*., 2011
In vitro	Drug resistance transfer	*L. infantum* - *L. major*	Coelho *et al*., 2012

2.13 *Leishmania* reference genome sequences

Table 11 lists the *Leishmania* spp. for which reference genomes have been sequenced. Strains are identified by WHO nomenclature HOST/COUNTRY/YEAR/STRAIN.

Table 11 Reference genomes of *Leishmania* spp.

Species & strain	Isolated from	Origin	Reference
L. major MHOM/IL/81/Friedlin	*Homo sapiens*	Israel	Ivens *et al*. (2005)
L. braziliensis MHOM/BR/75/M2904	*Homo sapiens*	Brazil	Peacock *et al*. (2007)
L. infantum MCAN/ES/98/LLM-877 (JPCM5)	*Canis familiaris*	Spain	Peacock *et al*. (2007)
L. mexicana MHOM/GT/2001/U1103 cl25	*Homo sapiens*	Guatemala	Rogers *et al*. (2011)
L. donovani MHOM/NP/2003/BPK282/0 cl4	*Homo sapiens*	Nepal	Downing *et al*. (2011)
L. amazonensis MHOM/BR/71973/M2269	*Homo sapiens*	Brazil	Real *et al*. (2013)

The *L. infantum* reference genome size was reported as 32.1 Mb (Peacock *et al*., 2007; Rogers *et al*., 2011). A comparison of the three hither-to sequenced genomes revealed 5 *L. major*-specific, 26 *L. infantum*-specific, and ~47 *L. braziliensis* specific genes (Peacock *et al*., 2007). Downing *et al*. (2011) published an assembled draft of the reference *L. donovani* genome strain, BPK282/0 cl4 originating from Nepal, which was chosen on the basis of being antimonial-sensitive and representative of the most common microsatellite group in South Asia (as identified by Alam *et al*., 2009). The genome size was 32.4 Mb, and

annotated by identifying orthologous genes from the *L. infantum* genome. Although *L. donovani* diversity in the region is low, the genome sequencing of an additional 16 clinical *L. donovani* strains form India and Nepal resolved a population structure beyond that found with MLMT.

2.14 Research needs: visceral leishmaniasis

The *Expert Committee Report on the Control of the Leishmaniases* (WHO, 2010b) describes laboratory research needs for VL, of which the most relevant to this project is 'research and development of rapid tests for the diagnosis of VL and tests for cure based on antigen and nucleic acid detection.'

3. AIMS AND OBJECTIVES

3.1 *Trypanosoma cruzi* and Chagas disease

Aim: To analyse molecular diversity within *T. cruzi*, identify lineage-specific antigenic epitopes, and apply them to determine serologically an individual's history of exposure to *T. cruzi* lineages. Objectives:

1) compare inter-lineage diversity in antigen genes by molecular analysis of the surface mucin TSSA, across a panel of strains encompassing *T. cruzi* genetic and ecological diversity [**ANNEX 1**];

2) development of a *T. cruzi* lineage-specific serology: design and use of synthetic peptides, based on the identified lineage-specific TSSA sequences, in ELISAs for differential serology with human chagasic sera [**ANNEX 2**].

3.2 *L. donovani* complex and visceral leishmaniasis

Aim: To determine the factors underlying the reported lower levels of sensitivity of rapid diagnostic tests among East African compared to South Asian human populations. Additionally, IgG subclass responses among VL patients of different clinical status are investigated as a potential biomarker. Objectives:

3) analyse the molecular diversity of diagnostic kinesin and HASPB antigens: PCR amplification and sequencing of kinesin and HASPB genes across a panel of East African *L. donovani* strains; comparative geographical analysis with reference to GenBank-accessible sequences [**ANNEX 3**];

4) compare Indian and Sudanese VL patient serological responses: production of whole cell lysates of representative Sudanese and Indian L*. donovani* strains; compare differential anti-*Leishmania* IgG responses between Indian and Sudanese VL patient cohorts [**ANNEX 4**];

5) analyse IgG subclass responses in Indian and Sudanese VL patients: compare IgG subclass profiles in patients with different clinical status [**ANNEX 5**];

6) adapt anti-*Leishmania* IgG1 detection to a lateral flow RDT for application at point-of-care [**ANNEX 5**].

4. MATERIALS AND METHODS

Descriptions of all reagents and protocols are described in the relevant publication(s) in Section 5 ANNEXES. Commonly used techniques are described here.

4.1 Parasite strains

Trypanosoma cruzi: Strains encompassing lineages TcI-TcVI used in this project are listed in ANNEX 1 Bhattacharyya *et al*., 2010 Table 1.

Leishmania donovani complex: Strains used in this project are listed in ANNEX 3 Bhattacharyya *et al*., 2013 Table 1.

4.2 Oligonucleotide sequences

Table 12 lists the oligonucleotides used as PCR and internal sequencing primers in ANNEXES 1, 2 & 3. All oligonucleotides synthesised by Eurofins MWG Operon.

Table 12 Oligonucleotides used as PCR and internal sequencing primers.

	Target	Forward/Reverse	Sequence (5' – 3')	Section
T. cruzi	TSSA	T5/ATG/E EMT5/A	CGGAATTCATGACTACGTGCCGTCT TTTGAGGAGGCTTCTGCTTC	ANNEX 1
	RNA binding protein, putative	*MenTcI For *MenTcI Rev	ATGCCACAATCGAAACCAAG TCACAACAAACGTTTGGCTG	ANNEX 2
L. donovani complex	Kinesin [Sequencing primer]	*LdonK39 for *LdonK39 rev *HASPB1intern	GAGCTCGCAACCGAGT CTGRCTCGCCAGCTCC (R= A/G) GATGAACGACTCTGCC	ANNEX 3
	HASPB [Sequencing primer]	*LdonHASPB1for *LdonHASPB1rev *HASPB1 intern	CATAAAACCACTGAGGC ATCTTCGTTCTTCTCCTG GATGAACGACTCTGCC	ANNEX 3

*= designed by the author for this project.

4.3 PCR, DNA Electrophoresis and cycle-sequencing.

PCR: General composition per 20 µl reaction volume in 0.2 ml capped tubes:

Reagent	amount	concentration
10x NH4 buffer	2 µl	1x
50mM $MgCl_2$	0.6 µl	1.5mM
2mM dNTPS	0.4 µl	40 µM
10pmol/ul Forward primer	1 µl	0.5 pmol/ µl (= µM)
10pmol/ul Reverse primer	1 µl	0.5 pmol/ µl (= µM)
BioTaq Polymerase	0.2 µl	1 U
H_2O	To volume	
Template DNA	1 -2 µl	

1 x cycle: 95 °C for 2 mins

25 cycles: 95 °C for 30 secs / Anneal temperature* for 30 secs / 72°C for 30-60 secs*

1x cycle: 72 °C for 10 mins

* varies according to the expected amplicon

DNA electrophoresis: DNA (amplicons) was added to loading buffer, and electrophoresed on a gel of appropriate percentage of agarose (Bioline), containing 0.2 µg/ml EtBr, in 1xTAE buffer. DNA size standard marker was also loaded on each gel. Following electrophoresis, the gel was visualised and image-captured under UV using the GeneGenius BioImaging System (Syngene).

Cycle sequencing: General composition per 10 µl reaction volume in 0.2 ml capped tubes:

Reagent	amount
BigDye® Terminator v3.1 RR-100	0.5 µl
5x dilution buffer	1.7 µl
3.2 pmol/ µl primer	1 µl
Template DNA	1-2 µl
H_2O	To volume

Following 25 x cycles, comprising steps at 96 °C for 30 secs, 50 °C for 20 secs, and 60 °C for 4 mins, the resulting extension products were purified. Reactions were transferred to 1.5 ml tubes on ice, containing 32 µl of 95% EtOH and 8 µl ddH_2O. Following 15 min incubation, the samples were spun at 13000 rpm in a bench top microfuge for 20 mins. The pellets were washed by 50 µl of 70 % EtOH, and dried at 90°C for 1 min. Sequencing was performed on an ABI3730 machine (Applied Biosystems). Sequencing analysis and alignments were performed using Bioedit software (Hall, 1999).

4.4 Peptide synthesis

'In-house' peptide synthesis was performed in collaboration with Dr A. Falconar, LSHTM. The automated systems used were 'SMPS350 Peptide Synthesiser' (Zinsser Analytic), and an 'Apex396 Peptide Synthesiser' (Aapptec). Additionally, peptides were produced commercially by Genosphere Biotechnologies. A detailed description of the synthesis and purification of peptides, from Dr Falconar, is given in ANNEX 2 Bhattacharyya *et al*., 2014. A brief overview of the synthesis process, based on the sequential addition of amino acid residues (initially protected at the α-amino and R side chains) to a solid phase support in the overall direction C → N, is given here (Figure 36).

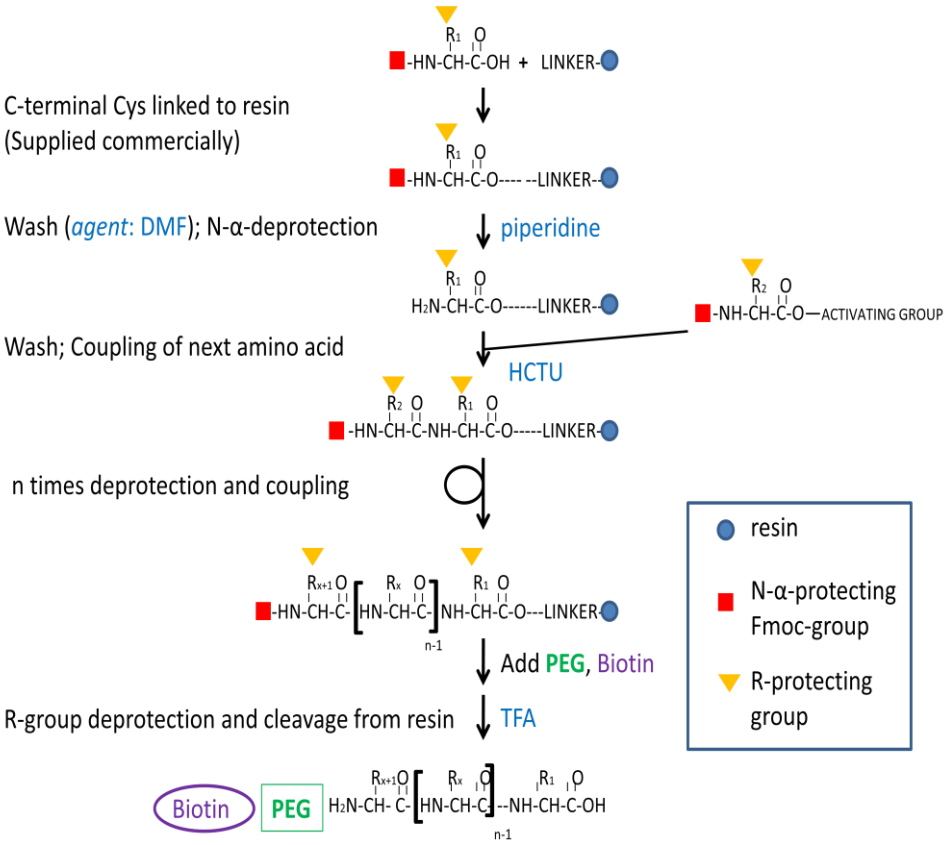


Figure 36 Overall peptide synthesis scheme. Adapted from White and Dörner (eds.), Synthesis Notes, Novabiochem Peptide Synthesis catalogue 2008/2009.

A C-terminal cysteine residue linked to resin was used to prime the synthesis of each peptide. After removal of the N-α-protecting group by piperidine, subsequent amino acids were coupled. DMF was used in washing steps. A PEG spacer was coupled after the last amino acid residue (always glycine), and a terminal biotin group was added to each peptide. R-side chain deprotection and cleavage from the resin was then done. Thus, the final composition of the synthesised peptide is depicted in ANNEX 2 Bhattacharyya *et al*., 2014 Figure 1A. Following cleavage from the resin, peptides were purified by reverse-phase HPLC. Eluted peptide fractions were freeze-dried and stored at -85 °C.

4.5 Sources of human samples

Chagas disease: Hospital das Clinicas, Goiânia, Brazil; Universidad Mayor de San Simon, Cochabamba, Bolivia; Universidad Nacional de Salta, Argentina; Universidad Central de

Venezuela, Caracas, Venezuela; Universidad de los Andes, Bogotá, Colombia; Pontificia Universidad Católica del Ecuador, Quito, Ecuador.

VL: Banaras Hindu University, Varanasi, India; University of Khartoum, Sudan.

4.6 ELISA

Chagas disease: Protocols for confirming boitinylation of synthetic peptides (a proxy for peptide integrity) and performing lineage-specific serology are given in ANNEX 2 Bhattacharyya *et al*., 2014

VL: Protocols for comparative serology of Indian and Sudanese VL plasma, and IgG subclass in different clinical states, are given, respectively, in ANNEX 4 Bhattacharyya *et al*., 2014 and ANNEX 5 Bhattacharyya *et al*., 2014.

5. PUBLICATIONS ARISING: First author

Trypanosoma cruzi and Chagas disease

ANNEX 1: Bhattacharyya T, Brooks J, Yeo M, Carrasco HJ, Lewis MD, Llewellyn MS, Miles MA (2010). Analysis of molecular diversity of the *Trypanosoma cruzi* trypomastigote small surface antigen reveals novel epitopes, evidence of positive selection and potential implications for lineage-specific serology. *Int J Parasitol* 40 921-928

ANNEX 2: Bhattacharyya T, Falconar A, Luquetti A, Cosatles J, Grijalva M, Lewis M, Messenger L, Tran T, Ramirez JD, Guhl F, Carrasco H, Diosque P, Litvinov S, Miles MA (2014). Development of peptide-based lineage-specific serology for chronic Chagas disease: geographical and clinical distribution of epitope recognition. *PLoS Negl Trop Dis* 8 e2892

Leishmania donovani complex and visceral leishmaniasis

ANNEX 3: Bhattacharyya T, Boelaert M, Miles MA (2013). Comparison of visceral leishmaniasis diagnostic antigens in African and Asian *Leishmania donovani* reveals extensive diversity and continent-specific polymorphisms. *PLoS Negl Trop Dis* 7 e2057

ANNEX 4: Bhattacharyya T, Bowes DE, El-Safi S, Sundar S, Falconar AK, Singh OP, Kumar R, Ahmed O, Boelaert M, Miles MA (2014). Significantly lower anti-*Leishmania* IgG responses in Sudanese versus Indian visceral leishmaniasis. *PLoS Negl Trop Dis* 8 e2675

ANNEX 5: Bhattacharyya T, Ayandeh A, Falconar A, Sundar S, El-Safi S, Gripenberg M, Bowes D, Thunissen C, Singh OP, Kumar R, Ahmed O, Eisa O, Saad A, Pereira SS, Boelaert M, Mertens P, Miles MA (2014). IgG1 as a potential biomarker of post-chemotherapeutic relapse in visceral leishmaniasis, and adaptation to a rapid diagnostic test. *PLoS Negl Trop Dis* 8 e3273

In preparation

Costales J, Kotton C, Zurita-Leal A, Garcia-Perez J, Llewellyn M, Messenger L, Bhattacharyya T, Burleigh B. *Trypanosoma cruzi* I chronic chagasic cardiomyopathy and Chagas disease reactivation in Boston, Massachusetts, USA.

Presented papers

Oral presentations and posters

PUBLICATIONS ARISING: *Trypanosoma cruzi* and Chagas disease

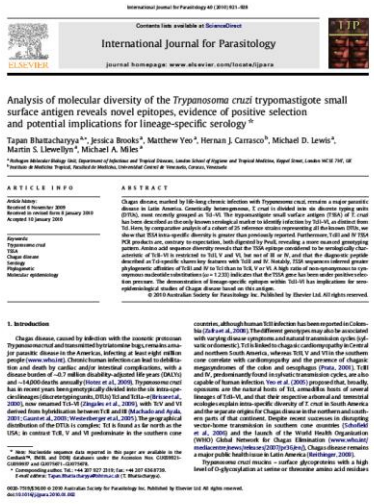
ANNEX 1: **Bhattacharyya T**, Brooks J, Yeo M, Carrasco HJ, Lewis MD, Llewellyn MS, Miles MA (2010). Analysis of molecular diversity of the *Trypanosoma cruzi* trypomastigote small surface antigen reveals novel epitopes, evidence of positive selection and potential implications for lineage-specific serology. *Int J Parasitol* 40 921-928

Key points, novel results and implications

- The *T. cruzi* surface mucin trypomastigote small surface antigen (TSSA) had been previously described as displaying sequence dimorphism between TcI and the then grossly-described TcII (now TcII-TcVI) lineages, and differential serology based on the antigenic properties of these sequences had led those authors to conclude that Chagas disease is due to infection by 'TcII' only (Di Noia *et al*., 2002).
- Here, the diversity of the TSSA gene was examined across a panel of *T. cruzi* isolates from TcI-TcVI lineages, encompassing the geographical and ecological range of this species.
- A greater diversity in the TSSA coding region, reflected in the predicted amino acid sequence, was identified. All TcI strains possessed their previously described sequence in the polymorphic region, whereas all TcII, TcV, and TcVI strains shared a common sequence, with TcV and TcVI strains possessing an additional haplotype coding for a single amino acid point substitution, not previously reported. TcIII and TcIV were revealed to contain their own lineage-specifc sequences, also reported here first.
- Analysis of the TSSA coding sequences among the panel inferred greater phylogenetic affinities of TcIII and TcIV to TcI than to TcII, TcV or TcVI, in agreement with published multilocus studies; a high ratio of non-synonymous to synonymous nucleotide substitutions ($\omega = 1.233$) indicated that the TSSA gene has been under positive selection pressure.
- Thus the demonstration here of a greater diversity of lineage-specific TSSA epitopes within TcII–TcVI has implications for seroepidemiological studies of Chagas disease based on this antigen.

Candidate's contribution:

The candidate performed all the PCRs, gel electrophoresis, DNA sequencing preparation, sequence alignment, and most of the RFLP assays, of the TSSA gene as described in this publication. The candidate prepared the first draft of the manuscript, which was accepted for publication by *Int J Parasitol* in January 2010 following academic peer review.



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International Journal for Parasitology

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Analysis of molecular diversity of the *Trypanosoma cruzi* trypomastigote small surface antigen reveals novel epitopes, evidence of positive selection and potential implications for lineage-specific serology ☆

Tapan Bhattacharyya, Jessica Brooks, Matthew Yeo, Hernan J. Carrasco, Michael D. Lewis, Martin S. Llewellyn, Michael A. Miles

ARTICLE INFO

ABSTRACT

1. Introduction

(Double-click on image to view embedded document)

The results described above were then applied to the design and use of synthetic peptides for *T. cruzi* lineage-specific serology, as described in the following section (ANNEX 2 Bhattacharyya *et al*., 2014).

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International Journal for Parasitology 40 (2010) 921–928

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Analysis of molecular diversity of the *Trypanosoma cruzi* trypomastigote small surface antigen reveals novel epitopes, evidence of positive selection and potential implications for lineage-specific serology ☆

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ARTICLE INFO

Article history:
Received 6 November 2009
Received in revised form 8 January 2010
Accepted 10 January 2010

Keywords:
Trypanosoma cruzi
TSSA
Chagas disease
Serology
Phylogenetic
Molecular epidemiology

ABSTRACT

Chagas disease, marked by life-long chronic infection with *Trypanosoma cruzi*, remains a major parasitic disease in Latin America. Genetically heterogeneous, *T. cruzi* is divided into six discrete typing units (DTUs), most recently grouped as TcI–VI. The trypomastigote small surface antigen (TSSA) of *T. cruzi* has been described as the only known serological marker to identify infection by TcII–VI, as distinct from TcI. Here, by comparative analysis of a cohort of 25 reference strains representing all the known DTUs, we show that *TSSA* intra-specific diversity is greater than previously reported. Furthermore, TcIII and IV *TSSA* PCR products are, contrary to expectation, both digested by PvuII, revealing a more nuanced genotyping pattern. Amino acid sequence diversity reveals that the TSSA epitope considered to be serologically characteristic of TcII–VI is restricted to TcII, V and VI, but not of III or IV, and that the diagnostic peptide described as TcI-specific shares key features with TcIII and IV. Notably, *TSSA* sequences inferred greater phylogenetic affinities of TcIII and IV to TcI than to TcII, V or VI. A high ratio of non-synonymous to synonymous nucleotide substitutions ($\omega = 1.233$) indicates that the TSSA gene has been under positive selection pressure. The demonstration of lineage-specific epitopes within TcII–VI has implications for sero-epidemiological studies of Chagas disease based on this antigen.

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1. Introduction

Chagas disease, caused by infection with the zoonotic protozoan *Trypanosoma cruzi* and transmitted by triatomine bugs, remains a major parasitic disease in the Americas, infecting at least eight million people (www.who.int). Chronic human infection can lead to debilitation and death by cardiac and/or intestinal complications, with a disease burden of ~0.7 million disability-adjusted life years (DALYs) and ~14,000 deaths annually (Hotez et al., 2009). *Trypanosoma cruzi* has in recent years been genotypically divided into the six intra-species lineages (discrete typing units, DTUs) TcI and TcII a–e (Brisse et al., 2000), now renamed TcI–VI (Zingales et al., 2009), with TcV and VI derived from hybridisation between TcII and III (Machado and Ayala, 2001; Gaunt et al., 2003; Westenberger et al., 2005). The geographical distribution of the DTUs is complex: TcI is found as far north as the USA; in contrast TcII, V and VI predominate in the southern cone countries, although human TcII infection has been reported in Colombia (Zafra et al., 2008). The different genotypes may also be associated with varying disease symptoms and natural transmission cycles (sylvatic or domestic). TcI is linked to chagasic cardiomyopathy in Central and northern South America, whereas TcII, V and VI in the southern cone correlate with cardiomyopathy and the presence of chagasic megasyndromes of the colon and oesophagus (Prata, 2001). TcIII and IV, predominantly found in sylvatic transmission cycles, are also capable of human infection. Yeo et al. (2005) proposed that, broadly, opossums are the natural hosts of TcI, armadillos hosts of several lineages of TcII–VI, and that their respective arboreal and terrestrial ecologies explain intra-specific diversity of *T. cruzi* in South America and the separate origins for Chagas disease in the northern and southern parts of that continent. Despite recent successes in disrupting vector-borne transmission in southern cone countries (Schofield et al., 2006) and the launch of the World Health Organization (WHO) Global Network for Chagas Elimination (www.who.int/mediacentre/news/releases/2007/pr36/en/), Chagas disease remains a major public health issue in Latin America (Reithinger, 2009).

Trypanosoma cruzi mucins – surface glycoproteins with a high level of O-glycosylation at serine or threonine amino acid residues

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the Accession Nos. GU059921–GU059937 and GU075671–GU075678.

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doi:10.1016/j.ijpara.2010.01.002

– comprise a large (>800) gene family (El-Sayed et al., 2005) believed to play a key role in host immune evasion and in maintaining infection (Buscaglia et al., 2006). The majority of mucin genes belong to the multi-gene sub-families TcMUC I and TcMUC II. Di Noia et al. (2002) assigned a bi-allelic single-copy gene to a third mucin family (TcMUC III), the two alleles of which were reported to correspond in distribution with lineages TcI and TcII–VI, respectively. Antisera raised against a recombinant protein product of this gene from strain CL Brener (TcVI) identified the native form expressed on the surface of cell-derived trypomastigotes (equivalent to bloodstream form trypomastigotes from mammalian hosts). The protein was thus named the trypomastigote small surface antigen (TSSA). They also reported: (i) PvuII digestion of a *TSSA* PCR product occurred only for TcI, not TcII–VI; (ii) the major antibody (Ab)-recognition epitope was restricted to a 10-amino acid region in TcII–VI (41-KPATGEAPSQ-50) where TcI versus TcII–VI differences clustered, with no immunological cross-reactivity between TSSA-I and TSSA-II isoforms. In a survey of chagasic patient sera from Argentina, Brazil and Chile, anti-TSSA antibodies were attributable only to the TcII–VI isoform, leading to the proposition that TSSA is the first serological marker to identify a *T. cruzi* lineage in human infection and that TcII–VI, not TcI, is the cause of Chagas disease. However, the Di Noia et al. (2002) study was based on a genotypically narrow range of *T. cruzi* strains, including those grossly categorised as TcII–VI, and on sera from a limited geographical area, as was later acknowledged (Buscaglia et al., 2006). Furthermore, TcI is known to cause severe and fatal Chagas disease with myocarditis in Venezuela (Miles et al., 1981a; Añez et al., 2004) and north-eastern Brazil (Teixeira et al., 2006).

Here, we determine the nucleotide (nt) and predicted amino acid sequence of a fragment of the *TSSA* gene across a panel of 25 *T. cruzi* strains representing a geographical and genotypic range that encompasses all described DTUs. A greater diversity is revealed than previously reported, including in the PvuII digest pattern, and in the region spanning the reported Ab-recognition epitope. The epitope considered specific for TcII–VI is shown to identify only TcII, V and VI. In addition, the peptide described as TcI-specific shares key features with TcIII and IV. We demonstrate that the bi-allelic description of the *TSSA* gene is not sufficient when all DTUs are examined, and that on the restricted basis of this gene, TcIII and IV share greater inferred phylogenetic affinities with TcI than with TcII, V and VI. The identification of lineage-specific epitopes within TcII–VI indicates a revised potential differential serology for epidemiological surveys of *T. cruzi* using this antigen.

2. Materials and methods

Table 1 lists the *T. cruzi* strains used in this study and their origins, all of which were biological clones. The PCR primers T5/ATG/E and EMT5/A, as described in Di Noia et al. (2002), were used to amplify an approximately 190 bp region of the *TSSA* gene from genomic DNA across the panel of strains listed in Table 1. Reactions comprised of 1× NH_4 reaction buffer, 1.5 mM $MgCl_2$ (Bioline, UK), 40 μM dNTPs (NEB, UK), 10 pmol of each primer and 1 U BioTaq DNA polymerase (Bioline). Amplification conditions were: one cycle at 94 °C for 3 min; 25 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s; and one cycle at 72 °C for 10 min. PCR products were digested using 2 U PvuII (Promega, UK) and separated by electrophoresis on 2.5% agarose gels (Bioline). DNA sequencing was achieved using a BigDye® Terminator v3.1 RR-100 kit (Applied Biosystems, UK) according to standard protocols. Sequence alignment was performed using BioEdit software (Hall, 1999), phylogenetic analysis using MEGA4 software (Tamura et al., 2007), and non-synonymous/synonymous nt substitutions per site ratios (dN/dS; ω) were calculated using SNAP software (Korber, 2000) employing the method of Nei and Gojobori (1986) with a Jukes-Cantor correction (http://hcv.lanl.gov/content/sequence/SNAP/SNAP.html).

3. Results

3.1. Alignment of TSSA gene fragment DNA sequences

Fig. 1A shows the nt polymorphisms within and between DTUs for the *TSSA* gene fragment across the panel of strains described in

Table 1
Panel of biological clones of *Trypanosoma cruzi* strains used in this trypomastigote small surface antigen (TSSA) comparison. Previous nomenclature for TcII–VI[IIa–e] is shown in brackets.

Discrete typing unit	Strain	Origin	Host	Reference
TcI	Sylvio X10/1	Belém, Brazil	*Homo sapiens*	Miles et al. (1978)
	Cutia cl1	Espiritu Santo, Brazil	*Dasyprocta agouti*	Brenière et al. (1998)
	Sp104 cl1	Region IV, Chile	*Triatoma spinolai*	Brenière et al. (1991)
	P209 cl93	Sucre, Bolivia	*H. sapiens*	Brenière et al. (1998)
	OPS21 cl11	Cojedes, Venezuela	*H. sapiens*	Brisse et al. (2000)
	92101601P cl1	Georgia, USA	*Didelphis marsupialis*	Barnabé et al. (2001)
TcII[IIb]	TU18 cl93	Tupiza, Bolivia	*Triatoma infestans*	Brenière et al. (1998)
	CBB cl3	Region IV, Chile	*H. sapiens*	Brenière et al. (1991)
	Mas cl1	Brasilia, Brazil	*H. sapiens*	Brisse et al. (2000)
	IVV cl4	Region IV, Chile	*H. sapiens*	Brenière et al. (1998)
	Esm cl3	São Felipe, Brazil	*H. sapiens*	Miles et al. (1977)
TcIII[IIc]	M5631 cl5	Selva Terra, Brazil	*Dasypus novemcinctus*	Miles et al. (1981b)
	M6241 cl6	Belém, Brazil	*H. sapiens*	Tibayrenc and Ayala (1988)
	CM17	Meta, Colombia	*Dasypus* sp.	Brisse et al. (2000)
	X109/2	Makthlawaiya, Paraguay	*Canis familiaris*	Chapman et al. (1984)
TcIV[IIa]	CanIII cl1	Belém, Brazil	*H. sapiens*	Miles et al. (1978)
	92122102R	Georgia, USA	*Procyon lotor*	Sturm et al. (2003)
	Dog Theis	Oklahoma, USA	*Canis familiaris*	Barnabé et al. (2001)
TcV[IId]	MN cl2	Region IV, Chile	*H. sapiens*	Brisse et al. (2000)
	Bug 2148 cl1	Rio Grande do Sul, Brazil	*T. infestans*	Souto et al. (1996)
	SO3 cl5	Potosi, Bolivia	*T. infestans*	Brenière et al. (1991)
	SC43 cl1	Santa Cruz, Bolivia	*T. infestans*	Tibayrenc and Miles (1983)
TcVI[IIe]	CL Brener	Rio Grande do Sul, Brazil	*T. infestans*	Brisse et al. (1998)
	P63 cl1	Makthlawaiya, Paraguay	*T. infestans*	Chapman et al. (1984)
	Tula cl2	Tulahuen, Chile	*H. sapiens*	Tibayrenc and Ayala (1988)

A

STRAIN	Nucleotide position 36	48	58	92	106-108	113	117	120	124	130	137	139	149	151	156	GenBank
TcI																
Silvio X10/1	G	C	T	G	---	C	C	G	A	G	G	A	C	T	A	GU059921
Cutia cl1	A	C	T	G	---	C	C	G	A	G	G	A	C	T	A	GU059922
Sp104 cl1	A	C	T	G	---	C	C	G	A	G	G	A	C	T	A	GU059923
P209 cl93	G	C	T	G	---	C	C	G	A	G	G	A	C	T	A	GU059924
OPS21 cl11	G	C	T	G	---	A	C	G	A	G	G	A	C	T	A	GU059925
92101601P cl1	G	C	T	G	---	C	C	G	A	G	G	A	C	T	A	GU059926
TcII[IIb]																
TU18 cl93	G	C	A	G	TCT	C	A	T	C	A	A	G	A	C	G	GU059928
CBB cl3	G	C	A	G	TCT	C	A	T	C	A	A	G	A	C	G	GU059929
Mas cl1	G	C	A	G	TCT	C	A	T	C	A	A	G	A	C	G	GU059930
IVV cl4	G	C	A	G	TCT	C	A	T	C	A	A	G	A	C	G	GU059931
Esm cl3	G	C	A	G	TCT	C	A	T	C	A	A	G	A	C	G	GU075675
TcIII[IIc]																
M5631 cl5	G	T	T	T	TCT	C	G	G	G	G	A	G	C	T	A	GU075673
M6241 cl6	G	T	T	T	TCT	C	G	G	G	G	A	G	C	T	A	GU059932
CM17	G	T	T	T	TCT	C	G	G	G	G	A	G	C	T	A	GU075674
X109/2	G	T	T	T	TCT	C	G	G	G	G	A	G	C	T	A	GU059933
TcIV[IIa]																
CanIII cl1	G	C	T	T	TCT	C	C	G	A	G	A	G	C	T	A	GU075671
92122102R	G	C	T	T	TCT	C	C	G	A	G	A	G	C	T	A	GU075672
Dog Theis	G	C	T	T	TCT	C	C	G	A	G	A	G	C	T	A	GU059927
TcV[IId]																
MN cl2	G	C	W	K	TCT	C	A	T	C	R	A	G	A	C	G	GU075677
Bug 2148 cl1	G	C	W	K	TCT	C	A	T	C	R	A	G	A	C	G	GU059934
SO3 cl5	G	C	W	K	TCT	C	A	T	C	R	A	G	A	C	G	GU059935
SC43 cl1	G	C	W	K	TCT	C	A	T	C	R	A	G	A	C	G	GU075676
TcVI[IIe]																
CL Brener	G	C	W	K	TCT	C	A	T	C	R	A	G	A	C	G	GU075678
P63 cl1	G	C	W	K	TCT	C	A	T	C	R	A	G	A	C	G	GU059936
Tula cl2	G	C	W	K	TCT	C	A	T	C	R	A	G	A	C	G	GU059937

B

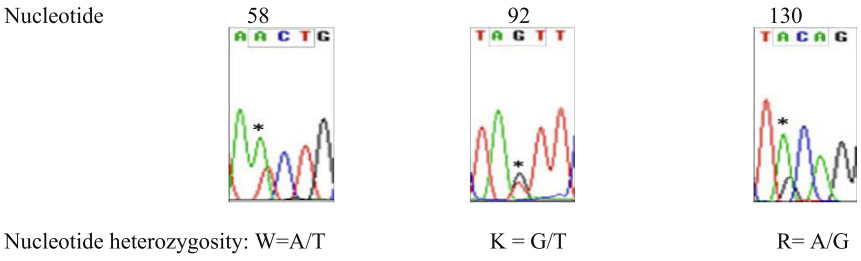

Fig. 1. Inter-discrete typing unit (DTU) diversity in the trypomastigote small surface antigen (*TSSA*) gene sequences. (A) Nucleotide polymorphisms within and between DTUs of the *TSSA* gene fragment across the panel of *Trypanosoma cruzi* reference strains. (B) Nucleotide heterozygosity in the CL Brener strain is represented by the degenerate symbols W, K and R. GenBank™ accession numbers refer to sequences derived in this study. Previous nomenclature for TcII–VI[IIa–e] is shown in brackets.

Table 1. For the hybrid strains from TcV and TcVI, certain nt peaks showed heterozygosity not recognised by the DNA sequencer basecaller software but observed on visual inspection of the electropherograms. These are represented by the data for the CL Brener strain (TcVI, Fig. 1B), and are depicted in the alignment by the degenerate symbols W (=A/T), K (=G/T) and R (=A/G). All sequences presented in Fig. 1A were derived in this study, and can be found in full under GenBank™ Accession Nos. GU059921–GU059937 and GU075671–GU075678; where sequences for certain strains had previously been submitted to GenBank™, these can be found with Accession Nos. AY367107–AY367113 and AF036443.

3.2. PvuII digest of TSSA PCR products

Sequencing of the *TSSA* PCR products revealed that the recognition site for PvuII (CAG↓CTG, nts 125–130) is present in the

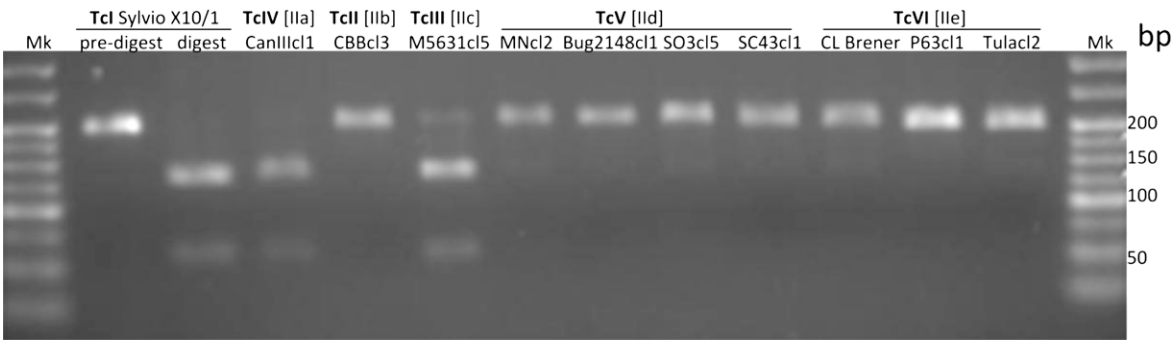

Fig. 2. PvuII digest of the trypomastigote small surface antigen (*TSSA*) PCR products. PCR products are shown after digestion, and pre-digested TSSA PCR product from Sylvio X10/1 is also shown. Previous nomenclature for TcII–VI[IIa–e] is shown in brackets.

Table 2
Summary of PvuII digests of the trypomastigote small surface antigen (*TSSA*) PCR products of reference strains, with additional strains used by [a]Di Noia et al. (2002). Previous nomenclature for TcII–VI[IIa–e] is shown in brackets.

Discrete typing unit	PvuII site presence (CAG↓CTG) or absence (CAGCTA)	Strains	Digest products (approx. size)
TcI	CAG↓CTG	Sylvio X10/1, 9, W250, X11161, X11541, CA-I/72[a]	125 bp + 65 bp
TcII[IIb]	CAGCTA	CBB cl3, Rita, Y[a]	190 bp (undigested)
TcIII[IIc]	CAG↓CTG	M5631 cl5, MA26X, M6241	125 bp + 65 bp
TcIV[IIa]	CAG↓CTG	CanIII, X11494, ERA	125 bp + 65 bp
TcV[IId]	CAG↓CTG CAGCTA	MN cl2, Bug 2148 cl1, SO3 cl5, SC43 Cl1	190 bp (undigested) + faint 125 bp
TcVI[IIe]	CAG↓CTG CAGCTA	CL Brener, P63 cl1, Tula cl2	190 bp (undigested) + faint 125 bp

sequences for TcI, III and IV but not TcII (CAGCTA). TcV and VI show heterozygosity at the 3′ nt of the PvuII site (nt 130, Fig. 1B), leading to the predicted presence of both a functional and abolished restriction site in these strains. Di Noia et al. (2002) used PvuII digestion of the *TSSA* PCR products to discriminate TcI from TcII–VI, yielding 122 and 68 bp fragments for TcI, indicating that TcII–VI were not digested. However, our wider in silico analysis indicates that the TcIII and IV PCR products can also be digested by this enzyme, TcII cannot, and lineages TcV and VI would be predicted to show a hybrid digest pattern. To investigate this further, *TSSA* PCR products representative of the DTUs including hybrid strains were digested by PvuII, and the resulting samples run on a 2.5% agarose gel as shown in Fig. 2.

In confirmation of the in silico comparison, TcII was not digested whereas TcI, III and IV produced two fragments of predictable size. Digests from the hybrid lineages gave the undigested PCR product plus a faint ~125 bp digest product (the ~65 bp not being readily visualised on ethidium bromide-stained gels), reflecting the patterns of the TcII and III parents. Further confirmation of these digest patterns for reference strains is shown in Table 2.

3.3. Amino acid diversity in TSSA

Fig. 3 shows the predicted amino acid sequences encoded by the *TSSA* gene fragment across the DTUs, including the amino acid changes resulting from the nt polymorphisms in the TcV and VI strains.

Several interesting features of the predicted amino acid sequences were observed. Firstly, using peptide scanning, the 10-amino acid region 41-KPATGEAPSQ-50 in TcII–VI was described as the Ab-recognition epitope that elicited the highest response with chagasic sera and allowed discrimination between TcI and TcII–VI (Di Noia et al., 2002). However, the amino acid sequences depicted in Fig. 3A reveal that this allele, although present in all TcII, V and VI strains examined here, is not found in either TcIII or IV. Second, in agreement with Di Noia et al. (2002), there is an absence of nt 106–108 in TcI compared with all of the TcII–VI strains (Fig. 1A), leading to an in-frame loss of residue Ser^{36} in the predicted amino acid sequence. This residue is part of the amino acid region identified in that publication as comprising a minor Ab-binding epitope. Thirdly, within the region encompassing residues 40–51, specific residues ($Lys^{40}Ala^{44}Pro^{50}Ser^{51}$) group TcI, III and IV whereas residues 39 and 42 split TcIII and IV. Last, for the hybrid strains TcV and VI the nt heterozygosity (shown in Fig. 1B) was predicted to result in amino acid changes in codons 20, 31 and 44.

3.4. Phylogenetic analysis based on TSSA sequences

Phylogenetic relationships of the *TSSA* nt sequences were analysed by reconstructing a Neighbour-Joining phylogeny based on *p*-distance (Fig. 4), taking into account the nt heterozygosity in the TcV and VI strains.

3.5. dN/dS ratio analysis of TSSA sequences

By comparing two DNA sequences and calculating the ratio of non-synonymous (changes amino acid, d*N*) and synonymous (does not change amino acid, d*S*) nt substitutions per site, a ratio of d*N*/d*S* greater than 1 can be used as an indicator that diversifying or positive selection has acted upon that sequence (Yang and Bielawski, 2000). Analysis of the *TSSA* sequences gave a d*N*/d*S* ratio $\omega = 1.233$.

4. Discussion

Understanding of the genetic diversity of *T. cruzi* has led to the description of intra-species lineages, or DTUs, and important in-

A

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                        10         20         30         40         50         60
TcI
Silvio X10/1      MTTCRLLCAL LALALCCCLS ACTTANGGST SSTPP-GTDK KTAAGGTPSP SGASSGEAEA SS
Cutia cl1         MTTCRLLCAL LALALCCCLS ACTTANGGST SSTPP-GTDK KTAAGGTPSP SGASSGEAEA SS
Sp104 cl1         MTTCRLLCAL LALALCCCLS ACTTANGGST SSTPP-GTDK KTAAGGTPSP SGASSGEAEA SS
P209 cl93         MTTCRLLCAL LALALCCCLS ACTTANGGST SSTPP-GTDK KTAAGGTPSP SGASSGEAEA SS
OPS21 cl11        MTTCRLLCAL LALALCCCLS ACTTANGGST SSTPP-GKDK KTAAGGTPSP SGASSGEAEA SS
92101601P cl1     MTTCRLLCAL LALALCCCLS ACTTANGGST SSTPP-GTDK KTAAGGTPSP SGASSGEAEA SS

TcII[IIb]
TU18 cl93         MTTCRLLCAL LALALCCCLT ACTTANGGST SSTPPSGTEN KPATGEAPSQ PGASSGEAEA SS
CBB cl3           MTTCRLLCAL LALALCCCLT ACTTANGGST SSTPPSGTEN KPATGEAPSQ PGASSGEAEA SS
Mas cl1           MTTCRLLCAL LALALCCCLT ACTTANGGST SSTPPSGTEN KPATGEAPSQ PGASSGEAEA SS
IVV cl4           MTTCRLLCAL LALALCCCLT ACTTANGGST SSTPPSGTEN KPATGEAPSQ PGASSGEAEA SS
Esm cl3           MTTCRLLCAL LALALCCCLT ACTTANGGST SSTPPSGTEN KPATGEAPSQ PGASSGEAEA SS

TcIII[IIc]
M5631 cl5         MTTCRLLCAL LALALCCCLS ACTTANGGST ISTPPSGTEK KAAAGEAPSP SGASSGEAEA SS
M6241 cl6         MTTCRLLCAL LALALCCCLS ACTTANGGST ISTPPSGTEK KAAAGEAPSP SGASSGEAEA SS
CM17              MTTCRLLCAL LALALCCCLS ACTTANGGST ISTPPSGTEK KAAAGEAPSP SGASSGEAEA SS
X109/2            MTTCRLLCAL LALALCCCLS ACTTANGGST ISTPPSGTEK KAAAGEAPSP SGASSGEAEA SS

TcIV[IIa]
CanIII cl1        MTTCRLLCAL LALALCCCLS ACTTANGGST ISTPPSGTDK KTAAGEAPSP SGASSGEAEA SS
92122102R         MTTCRLLCAL LALALCCCLS ACTTANGGST ISTPPSGTDK KTAAGEAPSP SGASSGEAEA SS
Dog Theis         MTTCRLLCAL LALALCCCLS ACTTANGGST ISTPPSGTDK KTAAGEAPSP SGASSGEAEA SS

TcV[IId]
MN cl2            MTTCRLLCAL LALALCCCL[T/S] ACTTANGGST [S/T]STPPSGTEN KPA[T/A]GEAPSQ PGASSGEAEA SS
Bug 2148 cl1      MTTCRLLCAL LALALCCCL[T/S] ACTTANGGST [S/T]STPPSGTEN KPA[T/A]GEAPSQ PGASSGEAEA SS
SO3 cl5           MTTCRLLCAL LALALCCCL[T/S] ACTTANGGST [S/T]STPPSGTEN KPA[T/A]GEAPSQ PGASSGEAEA SS
SC43 cl1          MTTCRLLCAL LALALCCCL[T/S] ACTTANGGST [S/T]STPPSGTEN KPA[T/A]GEAPSQ PGASSGEAEA SS

TcVI[IIe]
CL Brener         MTTCRLLCAL LALALCCCL[T/S] ACTTANGGST [S/T]STPPSGTEN KPA[T/A]GEAPSQ PGASSGEAEA SS
P63 cl1           MTTCRLLCAL LALALCCCL[T/S] ACTTANGGST [S/T]STPPSGTEN KPA[T/A]GEAPSQ PGASSGEAEA SS
Tula cl2          MTTCRLLCAL LALALCCCL[T/S] ACTTANGGST [S/T]STPPSGTEN KPA[T/A]GEAPSQ PGASSGEAEA SS
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Fig. 3. Inter-discrete typing unit (DTU) diversity in the trypomastigote small surface antigen (*TSSA*) protein. (A) Predicted amino acid sequences of the *TSSA* gene fragment of the *Trypanosoma cruzi* strains, including alternative residues encoded by the nucleotide heterozygosity shown in Fig. 1B. Colours indicate non-conserved residues. (B) Distribution of nucleotide polymorphisms leading to amino acid changes. Codons marked * showed presence of split peaks (see Fig. 1B). Previous nomenclature for TcII–VI[IIa–e] is shown in brackets.

sights have been gained into their geographic range, mammalian hosts and natural transmission cycles (Miles et al., 2009). However, a key goal remains to clarify the relationship between parasite genotype and clinical symptoms.

In this study, we analysed a fragment of the *TSSA* gene previously reported to contain a TcII–VI-specific immunogenic region, across a panel of *T. cruzi* strains representing the genotypic and geographical range of DTUs. We found a greater diversity at both the nt and amino acid levels than had been described. The PvuII digestion patterns revealed here also identify differences within the TcII–VI DTUs. To date, TSSA has been reported as the only *T. cruzi* antigen that provides a serological marker to identify TcII–VI infection. However, we demonstrate that the described TSSA-II epitope is present in only TcII, V and VI, not in TcIII and IV, and overall TSSA is significantly more diverse in amino acid structure than previously described, particularly in the major Ab-recognition epitope (41-KPATGEAPSQ-50). The steric polymorphism suggested here may also have immunogenic implications in the mature glycoprotein. The presence of $Ile^{31}Ala^{44}$ in TcIII and IV in place of $Ser^{31}Thr^{44}$ in TcII (Fig. 3A) would prohibit O-glycosylation at these residues and thus alter mucin formation. This may also have relevance if mucins have immunomodulatory effects on the persistence of parasite infection (Buscaglia et al., 2006). Fig. 1B shows that the TcV and VI strains display allelic heterozygosity at these codons, in agreement with their origin as the result of a hybridisation process between TcII and III. This is also reflected by the PvuII digests of these strains, where the heterozygosity at nt 130 results in the presence of a hybrid TcII/III digest pattern.

The phylogenetic relationships depicted in Fig. 4 indicate affinities between TcI, III and IV, supporting the revised *T. cruzi* nomenclature (Zingales et al., 2009), which considers these DTUs independently, not as sub-groups within the previous TcIIa-e nomenclature. The d*N*/d*S* ratio for *TSSA* was >1, showing that *TSSA* heterogeneity, reflected at the amino acid level, is likely a result of significant positive selection pressure favouring diversification of the protein. By comparison, house-keeping genes under negative selection pressure typically have d*N*/d*S* ratios <1, for example, for *T. cruzi* glucose-6-phosphate isomerase (*GPI*) sequences ($\omega = 0.112$) (M. Lewis, unpublished data). This lends further support to the suggestion that the TSSA divergence has functional significance and that TSSA has an important role in the host–parasite relationship. Furthermore, when its mammalian-stage restricted expression is also considered, this indicates that vertebrate immune systems have probably been the source of selection pressure. A postulated role may be found in the process of host cell invasion by *T. cruzi*, which is a multi-step process involving interaction of numerous parasite and host cell-surface molecules. Steps include host intracellular Ca^{2+} accumulation, lysosome recruitment to the

B

nucleotide/codon	Nucleotide polymorphism	Amino acid change	Distribution
58/20*	T	Ser	**TcI**, **III**[IIc], **IV**[IIa].
	A	Thr	**TcII**[IIb].
	T/A	Ser/Thr	**TcV**[IId], **VI**[IIe].
92/31*	G	Ser	**TcI**, **II**[IIb].
	T	Ile	**TcIII**[IIc], **IV**[IIa].
	G/T	Ser/Ile	**TcV**[IId], **VI**[IIe].
113/38	C	Thr	All except **TcI** OPS 21 cl1.
	A	Lys	**TcI** OPS21 cl1.
117/39	C	Asp	**TcI, IV**[IIa].
	A	Glu	**TcII**[IIb], **V**[IId], **VI**[IIe].
	G	Glu	**TcIII**[IIc].
120/40	G	Lys	**TcI, III**[IIc], **IV**[IIa].
	T	Asn	**TcII**[IIb], **V**[IId], **VI**[IIe].
124/42	A	Thr	**TcI, IV**[IIa].
	C	Pro	**TcII**[IIb], **V**[IId], **VI**[IIe].
	G	Ala	**TcIII**[IIc].
130/44*	G	Ala	**TcI, III**[IIc], **IV**[IIa].
	A	Thr	**TcII**[IIb].
	G/A	Ala/Thr	**TcV**[IId], **VI**[IIe].
137/46	G	Gly	**TcI**.
	A	Glu	**TcII**[IIb], **III**[IIc], **IV**[IIa], **V**[IId], **VI**[IIe].
139/47	A	Thr	**TcI**.
	G	Ala	**TcII**[IIb], **III**[IIc], **IV**[IIa], **V**[IId], **VI**[IIe].
149/50	C	Pro	**TcI, III**[IIc], **IV**[IIa].
	A	Gln	**TcII**[IIb], **V**[IId], **VI**[IIe].
151/51	T	Ser	**TcI, III**[IIc], **IV**[IIa].
	C	Pro	**TcII**[IIb], **V**[IId], **VI**[IIe].

Fig. 3 (*continued*)

cell surface and parasite internalisation (Andrews, 2002). As a bloodstream-stage expressed cell surface mucin, the diversity in TSSA could have implications for differential invasiveness between DTUs. The presence of distinct *TSSA* alleles in the genomes of TcV and VI strains may give them a putative selective advantage over non-hybrid strains. The well-characterised glycoproteins gp82 and gp85, both known to be involved with cell invasion (Yoshida, 2006), may also benefit from further study to evaluate the extent of stable polymorphism between lineages.

Work is now needed to examine whether the inter-DTU TSSA protein sequence variation described here can be exploited to discriminate further the DTUs in human infections, and enhance the understanding of the immunology and molecular epidemiology of the parasite across a wider geographical context. Di Noia et al. (2002) used a smaller set of *T. cruzi* strains than in this study, covering a narrower range of DTUs, and the chagasic sera used were from the southern cone countries only where TcII, V and VI are the DTUs most commonly found. We show here that the epitope previously identified as being characteristic of TcII–VI infection clearly only has the potential to detect serologically TcII, V and VI, so infection by TcIII or IV will be missed. Nonetheless, a serological test to distinguish TcII, V and VI from TcI, III and IV may still have utility. The genetic diversity and ecological niches of TcIII and IV are only recently beginning to be understood in sufficient detail. New hosts and a wider geographical distribution of TcIII have recently been reported (Llewellyn et al., 2009; Marcili et al., 2009a), while TcIV has been shown to be prevalent in several primate species and to be involved in oral transmission to humans in Amazonia (Marcili et al., 2009b).

A serological assay with the ability to discriminate immunodominant epitopes across the DTUs will allow identification of an individual's historic and extant lineage infections and aid investigation of clinical and epidemiological relationships. If appropriate successful tests can be devised, these may also be applicable to epidemiological tracking of the origins of infection in non-endemic areas caused as a result of transfusion, organ donation or vertical transmission routes. Screening of donor blood has begun recently in Europe (Gascon et al., in press) and the USA (www.cdc.gov/mmwr/preview/mmwrhtml/mm5607a2.htm).

This study demonstrates greater TcII–VI TSSA diversity than previously described in the nt sequence, PvuII-digest typing and amino acid diversity, crucially in the region described as the major Ab-binding epitope. By revealing the fuller extent of diversity and consequent implications for TSSA-based assays of *T. cruzi* infection,

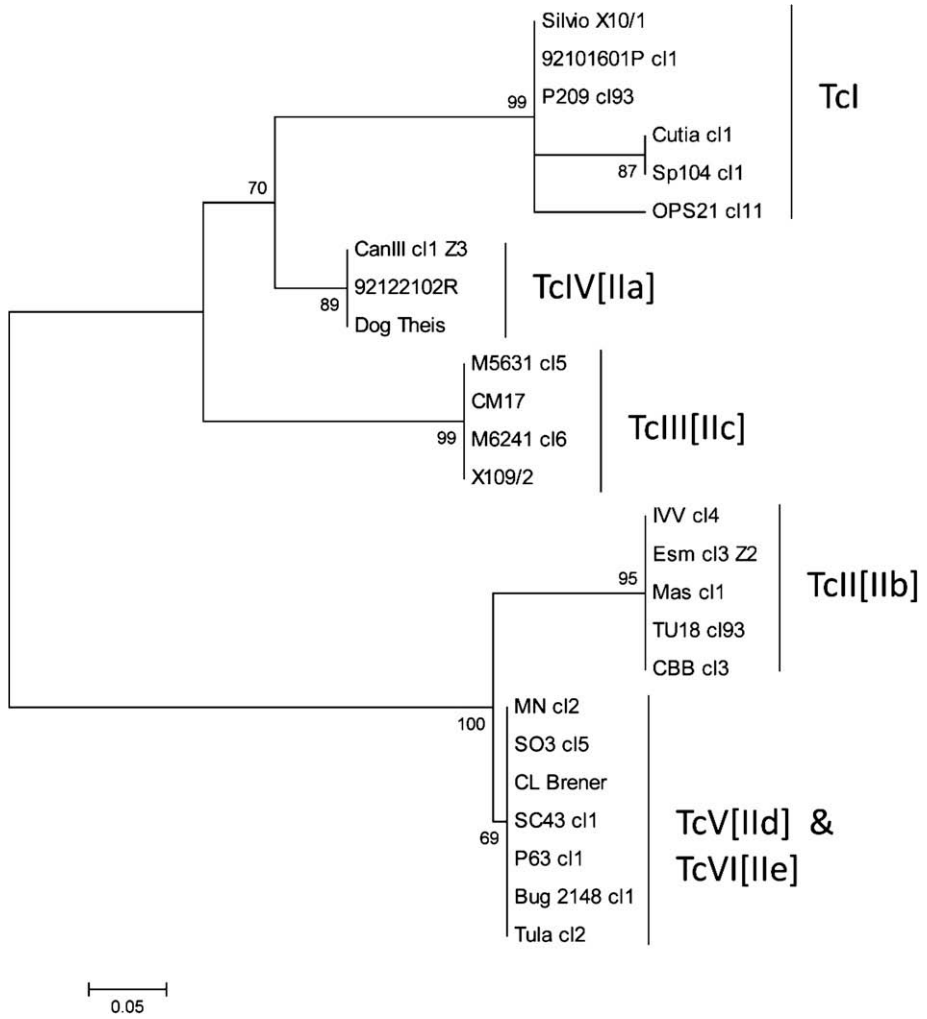

Fig. 4. Bootstrap test for Neighbour-Joining using trypomastigote small surface antigen (*TSSA*) gene sequences described in this study. Numbers indicate bootstrap values for the cluster nodes following 1,000 replicates. Previous nomenclature for TcII–VI[IIa–e] is shown in brackets.

a rationale is proposed for further immunological and molecular investigation of Chagas disease by the use of these lineage-specific epitopes for discriminatory serological assays.

Acknowledgements

This work was supported by the European Commission Framework Programme Project "Comparative epidemiology of genetic lineages of *Trypanosoma cruzi*" ChagasEpiNet, Contract No. 223034. We thank C. Barnabé (Institut de Recherche pour le Développement, Montpellier, France) for providing samples of *T. cruzi* genomic DNA.

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PUBLICATIONS ARISING: *Trypanosoma cruzi* and Chagas disease

ANNEX 2: **Bhattacharyya T,** Falconar AK, Luquetti AO, Costales JA, Grijalva MJ, Lewis MD, Messenger LA, Tran TT, Ramirez JD, Carrasco HJ, Diosque P, Garcia L, Litvinov SV, Miles MA (2014). Development of peptide-based lineage-specific serology for chronic Chagas disease: geographical and clinical distribution of epitope recognition. *PLoS Negl Trop Dis* 8 e2892

Key points, novel results and implications

- The identification of a much greater diversity within the polymorphic region of the trypomastigote small surface antigen (TSSA) among *T. cruzi* lineages has been described in ANNEX 1 Bhattacharyya *et al*., 2010.
- Here, synthetic lineage-specific epitope peptides based on this greater diversity were used in ELISA with sera from chagasic patients from throughout South America, in order to identify lineage-specific serological reactions.
- 79/113 (70%) of samples from Brazil, Bolivia, and Argentina recognised the TSSA epitope common to lineages TcII/TcV/TcVI, in agreement with previous descriptions of the distribution of these lineages. Comparison with clinical information showed that a higher proportion of Brazilian TSSApep-II/V/VI responders had ECG abnormalities than non-responders (38% vs 17%; $p<0.0001$).
- Among northern sera 4/20 (20%) from Ecuador reacted with TSSApep-II/V/VI. Also, 1/12 Venezuelan and 1/34 Colombian samples reacted with TSSApep-IV, in the first demonstration of a specific serological reaction against this lineage.
- In addition, a proposed TcI-specific epitope, described elsewhere (Mendes *et al*., 2013), was demonstrated here to be highly conserved across lineages and therefore not applicable to lineage-specific serology.
- These results demonstrate the considerable potential for synthetic peptide serology to investigate the infection history of individuals, geographical and clinical associations of *T. cruzi* lineages

Candidate's contribution:

The candidate performed and analysed all the ELISAs, designed the PCR primers and DNA analysis and alignment described in the submitted publication. The candidate prepared

the first draft of the manuscript, which was accepted for publication by *PLoS Negl Trop Dis* in April 2014 following academic peer review.

Development of Peptide-Based Lineage-Specific Serology for Chronic Chagas Disease: Geographical and Clinical Distribution of Epitope Recognition

Tapan Bhattacharyya[1*], Andrew K. Falconar[2], Alejandro O. Luquetti[3], Jaime A. Costales[4], Mario J. Grijalva[4,5], Michael D. Lewis[1], Louisa A. Messenger[1], Trang T. Tran[1], Juan-David Ramirez[6], Felipe Guhl[7], Hernan J. Carrasco[8], Patricio Diosque[9], Lineth Garcia[10], Sergey V. Litvinov[11], Michael A. Miles[1]

Abstract

Background: Chagas disease, caused by infection with the protozoan *Trypanosoma cruzi*, remains a serious public health issue in Latin America. Genetically diverse, the species is sub-divided into six lineages, known as TcI-TcVI, which have disparate geographical and ecological distributions. TcII, TcV, and TcVI are associated with severe human disease in the Southern Cone countries, whereas TcI is associated with cardiomyopathy north of the Amazon. *T. cruzi* persists as a chronic infection, with cardiac and/or gastrointestinal symptoms developing years or decades after initial infection. Identifying an individual's history of *T. cruzi* lineage infection directly by genotyping of the parasite is complicated by the low parasitaemia and sequestration in the host tissues.

Methodology/Principal Findings: We have applied here serology against lineage-specific epitopes of the *T. cruzi* surface antigen TSSA, as an indirect approach to allow identification of infecting lineage. Chagasic sera from chronic patients from a range of endemic countries were tested by ELISA against synthetic peptides representing lineage-specific TSSA epitopes bound to avidin-coated ELISA plates via a biotin labelled polyethylene glycol-glycine spacer to increase rotation and ensure each amino acid side chain could freely interact with their antibodies. 79/113 (70%) of samples from Brazil, Bolivia, and Argentina recognised the TSSA epitope common to lineages TcII/TcV/TcVI. Comparison with clinical information showed that a higher proportion of Brazilian TSSApep-II/V/VI responders had ECG abnormalities than non-responders (38% vs 17%; p<0.0001). Among northern chagasic sera 4/20 (20%) from Ecuador reacted with this peptide; 1/12 Venezuelan and 1/34 Colombian samples reacted with TSSApep-IV. In addition, a proposed TcI-specific epitope, described elsewhere, was demonstrated here to be highly conserved across lineages and therefore not applicable to lineage-specific serology.

Conclusions/Significance: These results demonstrate the considerable potential for synthetic peptide serology to investigate the infection history of individuals, geographical and clinical associations of *T. cruzi* lineages.

Introduction

Chagas disease (South American trypanosomiasis) is still considered to be the most important parasitic disease in Latin America, despite notable success with control of household infestation by the triatomine insect vectors. Up to 8 million people are estimated to be chronically infected with the causative agent *Trypanosoma cruzi*, of whom at least 30% are likely to develop chagasic cardiomyopathy, in some cases with megasyndromes of the intestinal tract [1,2]. Vector borne transmission is usually by

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Development of Peptide-Based Lineage-Specific Serology for Chronic Chagas Disease: Geographical and Clinical Distribution of Epitope Recognition

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Abstract

Background: Chagas disease, caused by infection with the protozoan *Trypanosoma cruzi*, remains a serious public health issue in Latin America. Genetically diverse, the species is sub-divided into six lineages, known as TcI–TcVI, which have disparate geographical and ecological distributions. TcII, TcV, and TcVI are associated with severe human disease in the Southern Cone countries, whereas TcI is associated with cardiomyopathy north of the Amazon. *T. cruzi* persists as a chronic infection, with cardiac and/or gastrointestinal symptoms developing years or decades after initial infection. Identifying an individual's history of *T. cruzi* lineage infection directly by genotyping of the parasite is complicated by the low parasitaemia and sequestration in the host tissues.

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Editor: Alain Debrabant, US Food and Drug Administration, United States of America

Received February 7, 2014; **Accepted** April 9, 2014; **Published** May 22, 2014

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Funding: This work was supported by the European Commission Framework Programme Project "Comparative epidemiology of genetic lineages of Trypanosoma cruzi" ChagasEpiNet, Contract No. 223034; http://cordis.europa.eu/fp7/home_en.html. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The epitope prediction was performed as a research collaboration with Sergey V. Litvinov of the company Aptum Biologics Ltd, without payment; none of the authors have any financial, non-financial, professional or personal conflicting interests; this collaboration does not alter our adherence to all PLOS NTDs policies on shared data and materials.

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Introduction

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Author Summary

Chagas disease remains a significant public health issue in Latin America. Caused by the single-celled parasite *Trypanosoma cruzi*, the main route of infection is via contact with contaminated faeces from blood-sucking triatomine bugs, but following successful insecticide spraying campaigns, congenital, food-borne, and transfusion/transplantation routes of infection have become more relevant. In the absence of successful chemotherapy, *T. cruzi* usually persists in the body for life, and in symptomatic cases may lead to death or debilitation by heart failure and/or gastrointestinal megasyndromes. As a species, *T. cruzi* displays great genetic diversity, and is subdivided into lineages called TcI - TcVI. Associating *T. cruzi* lineage with clinical symptoms is a key goal of Chagas disease research. Direct isolation and typing of *T. cruzi* from chronically infected patients is hampered by the sequestration of the parasite in host tissues. Identifying lineage-specific antibodies in serum provides an alternative approach to determining an individual's history of infection. Here, we performed lineage-specific serology using samples from a range of South American countries. We show that lineage-specific seropositivity is associated with geographical distributions and clinical outcome. These findings have wide implications for further diagnostics development and improved understanding of the epidemiology of Chagas disease.

contamination of mucous membranes or abraded skin with *T. cruzi* infected triatomine faeces and sporadic oral outbreaks occur due to triatomine contamination of food [3]. Infection can also be propagated by congenital transmission and blood or organ donation, and this may arise among migrant populations far beyond the endemic regions in Latin America [4].

The species *T. cruzi* is remarkably diverse genetically and is currently described as comprising six distinct lineages or discrete typing units (DTUs, TcI-TcVI) [5]. The six lineages have complex disparate but partially overlapping geographical and ecological distributions and are circumstantially associated with different epidemiological features [6,7]. TcI is the principal agent North of the Amazon, in association with chagasic heart disease but where megasyndromes are considered to be rare. TcII is one of three principal agents of Chagas disease in the Southern Cone region of South America, where chagasic cardiomyopathy, megaoesophagus and megacolon are found. TcIII is seldom isolated from humans but is widely distributed with the natural armadillo host *Dasypus novemcinctus*. TcIV is a sporadic secondary agent of Chagas disease in Venezuela [8]. TcV and TcVI, like TcII, are also agents of Chagas in the Southern Cone region, and are known to be relatively recent hybrids of TcII and TcIII [7,9].

Parasitological diagnosis in the acute phase of *T. cruzi* infection is by microscopy of fresh blood films, thin blood films, thick blood films or by haematocrit centrifugation and examination of the buffy coat, the latter being recommended particularly for congenital cases. In the chronic phase recovery of live organisms may be attempted by multiple blood cultures or xenodiagnosis with colony bred triatomine bugs but with limited sensitivities, or parasite DNA may be detectable by amplification.

Serological diagnosis of *T. cruzi* infection is usually performed by either indirect immunofluorescence (IFAT) or indirect haemaglutination (IHA) or enzyme-linked immunosorbent assay (ELISA), giving >94% sensitivity and specificity [2]. There are several commercially available diagnostic kits, including rapid lateral flow tests but sensitivities may not be equivalent, particularly when they are used in regions where non-homologous genetic lineages of *T. cruzi* are prevalent [8–10]. These serological methods give no information on the genetic lineage or lineages that a patient carries, and are not designed for that purpose.

A key objective of Chagas research therefore remains to follow up in detail the circumstantial evidence of a relationship between infecting *T. cruzi* lineage and the clinical outcome [6,7,11]. However, such analysis is complex and vulnerable to multiple confounders, including diversity of host susceptibility. Even if *T. cruzi* isolates can be recovered from the infected blood by parasitological diagnosis or if DNA can be amplified from blood, genotyping methods [12,13] do not provide an entire profile of the infecting lineages in an individual patient, because distinct *T. cruzi* lineages may be sequestered in the tissues [14]. An approach to overcoming this limitation is to identify infecting *T. cruzi* lineage in a more indirect way. One strategy to achieve this is by serological detection of antibodies that are produced in response to lineage-specific antigens.

Di Noia et al [15] described the trypomastigote small surface antigen (TSSA), encoded by a member of the *TcMUCIII* mucin gene family, expressed on the mammalian bloodstream trypomastigote stage of the *T. cruzi* life cycle. The authors reported that TSSA is dimorphic in sequence, with TSSA-I being present in TcI, and TSSA-II found in TcII-TcVI. On the basis of this finding the authors pioneered lineage-specific serology for Chagas disease through application of a TSSA-II recombinant antigen to serology with patients from the Southern Cone region of South America. Chagasic patients were only TSSA-II seropositive, which led to the suggestion that TcI could be benign. However, this suggestion was in conflict with the geographical predominance of TcI North of the Amazon and the acute and chronic clinical presentations of known TcI infections [16,17]. In subsequent publications *E. coli*-produced recombinant TSSA proteins have been used more widely for serology with humans and animals [18–23].

We have previously analysed *TSSA* diversity among a panel of *T. cruzi* isolates representing a broad geographical and ecological range of lineages TcI-TcVI [24]. We found a greater lineage-specific diversity than had previously been described. Lineages TcII, TcV, and TcVI were shown to share a common TSSA sequence. However, in both of the hybrid lineages TcV and TcVI we found that two TSSA alleles were present at an heterozygous locus within the polymorphic epitope: one haplotype was shared with TcII and in the second haplotype a Thr was replaced by Ala at position 44 of the protein. Lineage-specific TSSA sites were also found in TcIII and TcIV strains [24]. Cánepa et al [25] suggested a functional significance for this diversity in that the TcII/TcV/TcVI form of TSSA, but not the TcI form, has the property of binding surface receptor(s) and inducing signalling pathways in host cells prior to parasite internalisation.

Recently, Mendes et al [26] used a bioinformatic analysis of the reference genome of the TcVI strain CL Brener [27] to identify candidate peptides for differential screening with sera from mice experimentally infected with single, known *T. cruzi* lineages. A resultant peptide, derived from a putative RNA-binding protein, was reported to be applicable for TcI serology [26].

Here, we have used our expanded knowledge of the range of TSSA diversity to design and synthesise lineage-specific peptides. We assess the capacity of these peptides to provide antigens for lineage-specific serology by ELISA and thus reveal which lineages have infected individual patients during their lifetime. Furthermore, we examine the geographical and clinical distribution of recognition of the synthetic peptide epitopes. In addition, we also investigate the diversity of the gene coding for the peptide described [26] as applicable for TcI-specific serology.

Materials and Methods

Ethics statement

Human sera were collected as part of routine diagnostic examination, with local institutional ethical approvals, and in accord with EC ethical standards established as part of the ChagasEpiNet international collaboration. All human sera were anonymised and coded by letters and numbers that did not reveal patient identities. Production of mouse sera adhered to the European 3Rs policy of Refinement, Reduction and Replacement (99/167/EG: Council decision of 25/1/99), took place in authorised animal facilities by licensed staff in agreement with the European Directive 86/609/EEC, and with review and approvals under UK Home office regulations [Animals (Scientific Procedures) Act 1986; project licence number 70/6997 to the London School of Hygiene and Tropical Medicine].

Mouse and human sera

Mouse sera were from mice previously inoculated intraperitoneally with 10^6 organisms from stationary phase cultures containing infective metacyclic trypomastigotes, of known biological clones of *T. cruzi* representing the lineages. Sera were separated from whole mouse blood by allowing clotting at room temperature, overnight storage at 4°C, centrifugation at 12000×*g* for 10 mins and removal of the supernatant serum. Serum samples were stored 1:1 with glycerol at −20°C.

Human sera were from chronic cases of Chagas disease, confirmed by a combination of parasitological and serological diagnosis. As shown in Table 1, 113 samples were from the Southern Cone countries, Brazil, Bolivia and Argentina, and 66 samples were from countries North of the Amazon, Colombia, Ecuador, Venezuela, where TcI has been considered to predominate. Brazilian sera were from patients who had a positive parasitological diagnosis at the time of serum collection, together with a full clinical history, their geographical origin, age and sex. Institutes providing sera were: Hospital das Clinicas, Goiânia, Brazil; Universidad Mayor de San Simon, Cochabamba, Bolivia; Universidad Nacional de Salta, Argentina; Universidad Central de Venezuela, Caracas, Venezuela; Universidad de los Andes, Bogotá, Colombia; Pontificia Universidad Católica del Ecuador, Quito, Ecuador. Endemic healthy controls were from the Hospital das Clinicas, Goiânia, Brazil, and additional controls were 17 sera from Colombia that were serologically negative to *T. cruzi* lysate.

Synthesis of lineage-specific peptides

The synthetic peptides were prepared with an amino terminal biotin molecule linked via a polyethylene glycol-glycine spacer so that they could be bound to avidin-coated ELISA plates. Importantly, this method increased their rotation and ensured that each amino acid side chain could freely interact with antibodies, as opposed to being adsorbed onto the solid phase where some amino acid side chains would be unavailable, as discussed previously [28].

Design of the peptides was based on the *T. cruzi* TSSA lineage-specific amino acid sequences previously described [24]; chimeric peptides comprised by TSSA-I and TSSA-II sequences were also designed and synthesised (Results; Figure 1). Synthetic peptides were prepared at the 20 µM scale on 100–200 mesh-size Fmoc-Cys(Trt) Wang resin (0.5 mmol/g) (856006: Novabiochem, UK) using a Zinsser Analytic SMPS 350 (Zinsser Analytic, UK) or Advanced Chemtech Apex 396 (Advanced Chemtech, USA) robotic multiple peptide synthesizer. Aspartamide formation of aspartic acid residues was reduced by the use of OMpe-protected Fmoc-Asp(OMpe)-OH (852104: Novabiochem, UK). The coupling steps were performed using 0.5 M Fmoc-protected amino acids diluted in 6.76% (wt/vol) 1-hydroxybenzotriazole (HOBt)/dimethylformamide (DMF) (Activotec, UK/Rathburn Chemicals Ltd., UK) activated using 0.5 M N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate (HBTU) (851006: Novabiochem, UK) with 1M *N,N*-diisopropylethylamine (DIPEA) (Rathburn Chemicals Ltd., UK), while the deprotection steps were performed using 20% (vol/vol) piperidine/DMF (Rathburn Chemicals Ltd., UK). The carboxyl- and amino- regions flanking the core epitope sequences contained additional glycine (G) residues to increase rotation (high dihedral (ψ against φ) angles) of their carboxyl-terminal cysteine (C) residue and their amino-terminal spacer and molecular label. Their amino termini were labelled via a polyethylene glycol (PEG) spacer (Fig 1A) through sequential couplings with 0.5 M Fmoc-NH-$(PEG)_2$-COOH (13 atoms or 20 atoms) (851034 or 851031: Novabiochem, UK) followed by 0.5 M biotin (B4501: Sigma Aldrich, UK) using the more efficient coupling agent, 0.5 M N,N,N′,N′-tetramethyl-O-(7-azabenzotriazol-1-yl)uranium hexafluorophosphate (HATU) (851013: Novabiochem, UK) containing 1 M DIPEA. The final peptides were each washed 5 times with dichloromethane and then methanol (Rathburn Chemicals Ltd., UK) before being dried in a freeze-drier (Edwards, UK). Peptide cleavage was performed by reaction for 3–4 hours using 1% (wt/vol) phenol, 2% H_2O, 2.5% (vol/vol) triisopropyl silane (233781: Sigma Aldrich, UK) and 2% (vol/vol) 2,2′ (ethylenedioxy) diethanethiol (3,6-dioxa-1,8-octanedithiol (DODT)) (465178: Sigma Aldrich, UK) in trifluoroacetic acid (Rathburn Chemicals Ltd., UK) [29]. The cleaved peptides were then precipitated in cold (0°C) peroxide-free diethyl ether (Rathburn Chemicals Ltd., UK), centrifuged at 2,000×g; the supernatants were discarded and the precipitation and centrifugation steps were repeated twice. The peptides were then dried under a stream of anhydrous argon gas (BOC, UK) before being stored at −80°C.

For purification each peptide was subsequently dissolved in 500 µl of 2,2,2, trifluoroethanol (T63002: Sigma Aldrich, USA), made to 5.5 ml with 5% (vol/vol) far UV grade acetonitrile (Rathburn Chemicals Ltd., UK) in H_2O containing 0.1% (vol/vol) trifluoroacetic acid, and then subjected to reversed-phase HPLC using a 5 ml injection loop, a 5–95% acetonitrile gradient run at 9 ml/min over 30 min through an ACE C18–300 Å 250×21.2 mm preparative column (ACE-231-2520: Advanced Chromatography Technologies, UK) in a Beckman Gold preparative HPLC system (Beckman, USA). The main peaks, detected at a wavelength of 215 nm, were collected and freeze-dried before storage at −80°C.

Additionally, peptides TSSApep-II/V/VI, chimera TSSApep-I/-II, and MenTcI were also synthesised commercially (Genosphere Biotechnologies, Paris, France).

Purified peptides were prepared as 1 mg/ml stock solutions in PBS and the addition of biotin in the last coupling was assessed by ELISA. For this assay, 10 µg/ml of each peptide was prepared in 1× carbonate-bicarbonate coating buffer (15 mM Na_2CO_3, 34 mM $NaHCO_3$, pH 9.6) and added at 50 µl/well to 96-well ELISA plates (735–0465: Immulon 4HBX, VWR, UK). Plates were covered with an adhesive sheet and incubated overnight at 4°C. Following three washes with PBS containing 0.05% (vol/vol) Tween 20 (P7949: Sigma Aldrich, UK) (PBS/T), 200 µl/well blocking buffer (PBS/2% skimmed milk powder (Premier International Foods, Spalding, UK) was added and incubated at 37°C for 2 hrs. Following three washes, a 1:2000 dilution of peroxidase-labeled streptavidin (S2438: Sigma Aldrich, UK) in PBS/T containing 2% skimmed milk powder (PBS/T/M) was added at 50 µl/well, and incubated at 37°C for 1 hour. After washing six

Table 1. Geographical distribution of antibody responses to lineage-specific synthetic peptides, as determined by ELISA.

		TSSA peptide reaction								
		Lineage-specific						Chimera		Non-specific[g]
	n	I	II/V/VI	III	IV	V/VI	Non-reactive	I/II	II/I	
Brazil	98[a]	1[b]	67	1[b]	1[b]	11/67 of TcII/V/VI	28	9/67 of TcII/V/VI[c]	55/67 of TcII/V/VI	2
Bolivia	10	0	9	0	0	1/9 of TcII/V/VI	0	0/9 of TcII/V/VI	9/9 of TcII/V/VI	1
Argentina	5	1[d]	3	1[d]	1[d]	0/3 of TcII/V/VI	1	0/3 of TcII/V/VI	3/3 of TcII/V/VI	0
Colombia*	34	0	0	1[e]	1[e]	0	33	ND	ND	0
Ecuador	20	0	4	0	0	1/4 of TcII/V/VI	16	2/4 of TcII/V/VI[c]	4/4 of TcII/V/VI	0
Venezuela	12	0	0	1[f]	1[f]	0	10	1/1[f]	0	1
EHC (Brazil)	7	0	0	0	0	0	7	0	0	0
TOTAL	**186**	**2**	**83**	**4**[h]	**4**[h]	**13 of 83 of all TcII/V/VI**	**95**	**11 of the 83 TcII/V/VI positives & 1 of the 4 TcIV positives**	**71 of the 83 TcII/V/VI positives**	**4**

EHC = Endemic healthy controls (* a further 17 Colombian sera that were serologically negative with the lysate were included in the peptide ELISAs as additional controls); ND = not determined.

[a]these 98 comprised 1 sample from each of 90 patients, plus 2 paired samples from each of 4 patients. All eight paired samples reacted with TSSApep-II/V/VI, and are included within the 67 Brazilian reactors to this peptide. 1 set of these pairs also reacted with TSSApep-V/VI.

[b]same sample, which did not react with TSSApep-II/V/VI, TSSApep-V/VI or chimeras.

[c]these 9 samples also reacted with chimera TSSA-II/-I peptide.

[d]same sample, which did not react with TSSApep-II/V/VI, TSSApep-V/VI or chimeras.

[e]same sample.

[f]same sample, which did not react with TSSApep-I, TSSApep-II/V/VI, TSSApep-V/VI or chimera TSSApep-II/-I.

[g]non-specific binding; see text.

[h]in each case the same sample reacted with TSSApep-III and TSSApep-IV.

doi:10.1371/journal.pntd.0002892.t001

A

B

PEPTIDE	AMINO ACID SEQUENCE
TSSApep-I	GTDKKTAAGGTPSPSG
TSSApep-II/V/VI	GTENKPATGEAPSQPG
TSSApep-III	GTEKKAAAGEAPSPSG
TSSApep-IV	GTDKKTAAGEAPSPSG
TSSApep-V/VI	GTENKPAAGEAPSQPG
Chimera TSSApep-I/-**II**	GTDKKTA**TGEAPSQPG**
Chimera TSSApep-II/-**I**	**GTENKPATGE**TPSPSG

Figure 1. TSSA provides potential epitopes that are *T. cruzi* lineage-specific. [A] Components of the peptides synthesised: N-terminal biotinylation; PEG spacer; Gly; the lineage-specific sequence; C-terminal Cys. [B] Amino acid sequences of the *T. cruzi* lineage-specific TSSA potential epitopes in the synthetic peptides (TSSApep-), with polymorphic residues underlined; for the two chimeric peptides the TSSA-II residues are shown in bold.
doi:10.1371/journal.pntd.0002892.g001

times with PBS/T, 50 mM phosphate/citrate buffer (pH 5.0) containing 2 mM *o*-phenylenediamine HCl (P1526: Sigma Aldrich, UK) and 0.005% (vol/vol) H_2O_2 (216763: Sigma Aldrich, UK) was added at 50 µl/well and the plates were incubated in the dark at room temperature for 10 minutes. The substrate reactions were then stopped by the addition of 2M H_2SO_4 (25 µl/well) and the absorbance values were determined at a wavelength of 490 nm (MRX, Dynatech, USA).

Production of whole-cell lysate antigen

T. cruzi was cultured as previously described [30]. For production of lysate antigen, mid-to-late log phase cultures of a TcII strain (IINF/PY/00/Chaco23cl4) of *T. cruzi* were centrifuged at 800×*g* for 10 mins at 4°C in an Allegra X-15R benchtop centrifuge (Beckman Coulter, UK). After washing in PBS, cell pellets were subjected to 3 cycles of flash-freezing in liquid nitrogen and thawing in a cold water bath. Cell lysates were then sonicated for 3×30 sec, with intervals on ice, using a Soniprep 150 sonicator (MSE), at 12 µ amplitude. Sonicated lysates were centrifuged at 13000 rpm for 1 min, and the supernatant used as antigen in ELISA. Protein concentration was determined using the BCA Protein Assay kit (PN23227: Fisher Scientific, UK).

Lineage-specific peptide ELISA

Immulon 4HBX 96-well flat bottomed ELISA plates were coated with 1 µg/100 µl/well of avidin (A9275: Sigma, UK) diluted in 1× carbonate-bicarbonate coating buffer for binding to lineage-specific peptide, and in separate wells coating was with TcII *T. cruzi* lysate at 0.2 µg/100 µl/well to act as a serologically positive control for each sample. Plates were covered with an adhesive sheet and incubated overnight at 4°C. The following day, unbound avidin and lysate were removed, the plate washed three times with wash buffer PBS/T, then wells were blocked with 200 µl blocking buffer PBS/T/M at 37°C for 2 hrs. Following three washes, 1 µg/100 µl/well TSSA lineage-specific peptide in PBS/T/M was incubated with the avidin-coated wells at 37°C for 1 hr. Following three washes, 100 µl/well of a 1:200 dilution of serum in PBS/T/M was added and incubated at 37°C for 1 hr. Following six washes, 100 µl/well of donkey anti-human IgG (H+L)-HRP (709-035–149: Jackson Immunoresearch, Pennsylvania, USA), diluted 1:5,000 in PBS/T/M was added, and incubated at 37°C for 1 hr. Following six washes, plates were developed and read as described above, except that the volumes were 100 µl for substrate and 50 µl for 2M H_2SO_4. Replica plates were run in duplicate simultaneously.

Statistical analysis

Cut-off values for ELISAs with human sera and peptides were calculated from the mean plus 3 standard deviations compared to the endemic healthy controls from Goiânia, Brazil. Statistical analysis (2-tailed unpaired t-test) on the Brazilian TSSApep-II/V/VI seropositives and non-responders was performed using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, California, USA).

Analysing diversity of the putative TcI epitope

We designed PCR primers MenTcI FOR (5′ ATGCCACAATCGAAACCAAG 3′) and MenTcI REV (5′ TCACAACAAACGTTTGGCTG 3′) (synthesised by Eurofins MWG Operon, Germany) to amplify the whole open reading frame (ORF) of the putative RNA–binding protein (Tc00.1047 053511837.129) which was described as containing an epitope and corresponding peptide applicable for TcI serology [26]. *T. cruzi* strains, from which genomic DNA was used as amplification template, are listed in Table 2. Amplification reactions were performed in a total volume of 20 µl, and comprised of 1×NH_4 reaction buffer supplemented with 1.5 mM $MgCl_2$ (Bioline, UK), 200 mM dNTPs (New England Biolabs, UK), 10 pmol of each primer, and 1 U BioTaq DNA polymerase (Bioline). Amplification conditions were: 1 cycle of 94°C, 3 mins; 25 cycles of 94°C for 30 secs, 55°C for 30 secs, 72°C for 30 secs; 1 cycle of 72°C for 10 mins. Five microliters of the PCR reaction were analysed by electrophoresis on 1.5% agarose gels (Bioline); amplification products were purified from the remaining reaction by precipitation with an equal volume of isopropanol at room temperature, followed by washing with 70% EtOH, air-drying and resuspension in ddH_2O. Bi-directional DNA sequencing, using each PCR primer separately at 3.2 pmol, was achieved using a BigDye

Table 2. *T. cruzi* strains used here for comparative analysis of the ORF containing the reported TcI-applicable peptide (GenBank accession numbers refer to sequences determined here).

Lineage	Strain	Origin	Host/vector	Tc00.1047053511837.129 [26][a]	GenBank
				E N S A N P P P P D R S L P T P	
TcI	MHOM/BR/78/Sylvio-X10/1	Belém, Brazil	*Homo sapiens*	. . . . .	KJ395471
	MHOM/PE/00/SaxP18	Majes, Peru	*Homo sapiens*	. . . . .	KJ395472
	MPHI/BO/00/SJM41	Beni, Bolivia	*Philander opossum*	. . . . .	KJ395473
	MDID/BO/00/SMA2	Beni, Bolivia	*Didelphis marsupialis*	. . . . .	KJ395474
	MDID/BO/00/SJM37	Beni, Bolivia	*Didelphis marsupialis*	. . . . .	KJ395475
	MPHT/BO/00/COTMA47	Cotopachi, Bolivia	*Phyllotis ocilae*	. . . . .	KJ395476
TcII	MHOM/CL/00/IVV	Cuncumen, Chile	*Homo sapiens*	. . . . .	KJ395477
	MHOM/BR/00/Y	São Paulo, Brazil	*Homo sapiens*	. . . . . $^{S}_{T}$.	KJ395478
	MHOM/CL/00/CBB	Region IV, Chile	*Homo sapiens*	. . . . . $^{S}_{T}$.	KJ395479
	IINF/BO/00/Tu18	Tupiza, Bolivia	*Triatoma infestans*	. . . . .	KJ395480
	IINF/PY/00/Chaco23	Chaco, Paraguay	*Triatoma infestans*	. . . . .	KJ395481
	IINF/PY/00/T655	Chaco, Paraguay	*Triatoma infestans*	. . . . .	KJ395482
TcIV	IINF/AR/00/LHVA	Chaco, Argentina	*Triatoma infestans*	. . . . .	KJ395483
TcV	IINF/CL/00/Vinch101	Limari, Chile	*Triatoma infestans*	. . . . .	KJ395484
	MHOM/BO/00/92:80	Santa Cruz, Bolivia	*Homo sapiens*	. . . . .	KJ395485
	IINF/BR/00/Bug2148	Rio Grande do Sul, Brazil	*Triatoma infestans*	. . . . .	KJ395486
	IINF/PY/00/Para6	Paraguari, Paraguay	*Triatoma infestans*	. . . . . A . . A .	KJ395487
TcVI	MHOM/BR/00/CL Brener	Rio Grande do Sul, Brazil	*Triatoma infestans*	. . . . .	KJ395488
	MHOM/BO/00/P251	Cochabamba, Bolivia	*Homo sapiens*	. . . . .	KJ395489
	IINF/PY/00/Chaco17	Chaco, Paraguay	*Triatoma infestans*	. . . . .	KJ395490
	IINF/PY/00/Chaco9	Chaco, Paraguay	*Triatoma infestans*	. . . . .	KJ395491
	IINF/AR/00/EPV20-1	Chaco, Argentina	*Triatoma infestans*	. . . . .	KJ395492
	IINF/CL/00/VFRA	Francia, Chile	*Triatoma infestans*	. . . . .	KJ395493

[a]. = no amino acid change.

doi:10.1371/journal.pntd.0002892.t002

Terminator v3.1 RR-100 kit (Applied Biosystems, UK) according to standard protocols. Sequence alignment was performed using BioEdit software [31]. In parallel, the coding region of the TSSA gene containing lineage-specific sequences was also sequenced, as described previously [24], to confirm lineage identity.

Linear B-epitope profiling

Computer analysis of the TSSA-I and the TSSA-II/V/VI common epitope was performed using EpiQuest-B software (v 2.1.17, Matrix B7.1) from Aptum Biologics Ltd (Southampton, Hampshire, UK). The algorithm of the program allows prediction of potential linear B-epitopes and their immunogenicity. The data were used in graphical format.

Accession numbers

Nucleotide sequences derived in this manuscript are available under GenBank accession numbers KJ395471 - KJ395493.

Results

TSSA provides potential epitopes specific for each *T. cruzi* lineage

The structures and sequences of the peptides synthesised, indicating the lineage-specific amino acids, are shown in Figure 1, as based on the comparisons of diversity previously described [24]. In addition to the peptides representing single lineages we synthesised two chimeric peptides, one with TSSA-I residues at the N terminus and TSSA-II residues at the C terminus, and the second with TSSA-II at the N terminus and TSSA-I at the C terminus (Figure 1B).

Consistent with the known extensive genomic divergence between TcI and TcII, eight residues differed between their TSSA potential epitopes. Five and six residues separated TSSA-II from TSSA-III and TSSA-IV, respectively. Four residues distinguished TSSA-I from TSSA-III and two residues separated TSSA-I from TSSA-IV, in accord with their somewhat greater affinity with TcI. A single residue differed between the TSSA-II haplotype shared by TcII, TcV and TcVI and the second haplotype present at the heterozygous locus in the hybrids TcV and TcVI.

Synthetic peptides are recognised by serum antibodies

Sera from mice experimentally infected with biological clones of TcII, TcV and TcVI strains recognised TSSApep-II/V/VI in serology by ELISA, and sera from TcIII and TcIV murine infections reacted with the corresponding TSSA peptides (Bhattacharyya et al, in preparation), encouraging the evaluation described here of the diagnostic potential of all the synthetic peptides with sera from patients with chronic Chagas disease.

Figure 2 shows examples of ELISA plates with *T. cruzi* lysate and lineage-specific synthetic peptides as antigens. Sera from normal healthy endemic controls did not react with the *T. cruzi* lysate or with any of the synthetic peptides. Without exception all

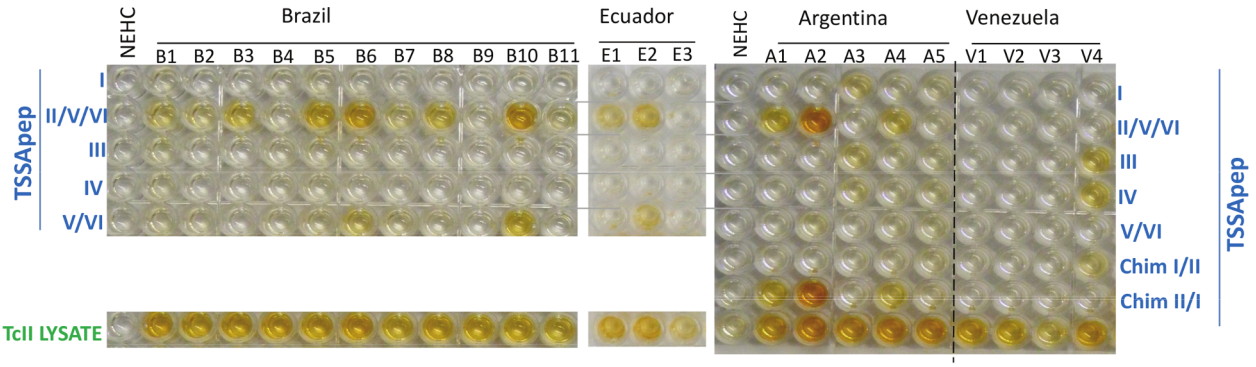

Figure 2. Chagasic sera recognise TSSA lineage-specific peptides. Lineage-specific peptides or lysate were added to rows of the ELISA plate as indicated. ELISA plates showing: recognition of TSSApep-II/V/VI and TSSApep-V/VI, among Brazilian, Ecuadorean and Argentine sera; recognition of TSSApep-I and TSSApep-IV by an Argentine serum and a Venezuelan serum; recognition of chimera TSSApep-II/I by Argentine sera. All patients were seropositive with *T. cruzi* lysate. NEHC = non-endemic healthy control.
doi:10.1371/journal.pntd.0002892.g002

sera from patients with chronic Chagas disease recognised the *T. cruzi* TcII lysate antigen preparation. Figure 2 also provides examples of sera from Brazil, Argentina and Ecuador that recognised TSSApep-II/V/VI, indicative of infection with TcII,TcV or TcVI. A positive result for the epitope derived from the TcV/VI specific haplotype indicates definite infection with TcV or TcVI. Some of these sera (e.g. B6 & B10) reacted with both TSSApep-II/V/VI *and* TSSApep-V/VI representing the haplotype restricted to TcV and TcVI, indicating infection with a hybrid strain, possibly in conjunction with a TcII infection. Recognition of the TcV and TcVI restricted epitope was never seen in the absence of recognition of the TSSApep-II/V/VI.

A Venezuelan serum (V4 in Figure 2) recognised TSSApep-IV, consistent with the known presence of TcIV as a secondary agent of Chagas disease in Venezuela [16]. However, this serum also bound to TSSApep-III, which only differs by 2 of 16 residues.

An antibody response to TSSApep-I was exceptional, only two sera were reactive from the entire set of samples (Table 1) of which one weak reactor (Argentina A3) is shown in Figure 2.

Chimera TSSApep-I/-II and chimera TSSApep-II/-I were designed to determine whether the antigenic epitope resided at the N or C terminus of the peptides. Chimera TSSApep-II/-I was recognised by 71/83 TSSApep-II/V/VI reactive sera, as demonstrated for example by Argentine patients A1, A2 and A4 (Figure 2). In comparison, only 11/83 recognised the chimera TSSApep-I/-II, indicative that, although not precisely mapped, the dominant region of the epitope lies towards the N terminus of the peptide and that in some patients the N terminus is adequate to provide a detectable epitope. A single TSSApep-IV/TSSApep-III positive serum also recognised the chimera TSSApep-I/-II peptide.

Four of 186 samples responded to all wells containing peptides; these were demonstrated to bind non-specifically to avidin in the absence of peptide, but not to cross react with milk proteins (data not shown).

Rare recognition of the TSSA-II/V/VI common peptide in northern South America

The 186 sera from patients with chronic Chagas disease spanned a geographical range from Argentina to Venezuela. Three Southern Cone countries were included, where TcII, TcV and TcVI have been reported to be endemic, and three countries from northern South America, where TcI is considered to predominate. A summary of the geographical distribution of the antibody responses to all the lineage-specific synthetic peptides is shown in Table 1. Of the sera recognizing TSSApep-II/V/VI, 79 out of 83 were from the Southern Cone countries and four were from Ecuador. Of these 83 sera, 13 sera also recognised TSSApep-V/VI, 12 from Southern Cone countries and one of the four sera from Ecuador, indicating presence of TcV or TcVI, possibly with TcII co-infection. Independently of the lineage-specific peptides, we also examined the response to two different chimera peptides, each comprising different combinations of sequences from TSSApep-I and TSSApep-II/V/VI. Of the Bolivian, Ecuadorean and Argentine sera which reacted with TSSApep-II/V/VI, all reacted with chimera TSSApep-II/-I, but only two samples (Ecuadorean) also reacted with chimera TSSApep-I/-II. In the case of Brazilian samples, of the 67 that reacted with TSSApep-II/V/VI, 55 reacted with chimera TSSApep-II/-I, and of these 55, 9 also reacted with chimera TSSApep-I/-II. Only one sample (Venezuelan) reacted with chimera TSSApep-I/-II but not with TSSApep-II/V/VI or chimera TSSApep-II/-I. TSSApep-I failed to detect antibodies, regardless of origin of the chagasic sera, with the exception of two sera, one each from Brazil and Argentina. Four sera recognised both TSSApep-IV and TSSApep-III, consistent with cross-reaction due to the close similarity between these epitopes.

The country by country distribution of antibody recognition of the peptides is given in Table 1. ELISA cut-offs and absorbance values for each lineage-specific peptide are shown in Figure 3. Each data point represents the mean A_{490} readout of duplicate assays of the serum sample with the lineage specific peptides. In Figure 3, the samples giving the highest reading for TSSApep-III from Colombia and Venezuela are the same samples that recognised TSSApep-IV.

Antibodies to the TcII/TcV/TcVI peptide are more frequent among symptomatic Brazilian patients

60/63 of the Brazilian patients with chronic Chagas disease who were seropositive against TSSApep-II/V/VI had detailed clinical evaluation, and of these 60 patients, 23 (38%) had ECG abnormalities typical of Chagas disease. 23/28 patients seronegative for TSSApep-II/V/VI also had detailed clinical evaluation, but in contrast only 4 of these latter, different 23 patients had such ECG abnormalities ($p<0.0001$).

Novel bioinformatic algorithms predict highly antigenic residues

The sequences coding for the TSSA proteins containing the TSSApep-I and TSSApep-II/V/VI epitopes were subjected to a

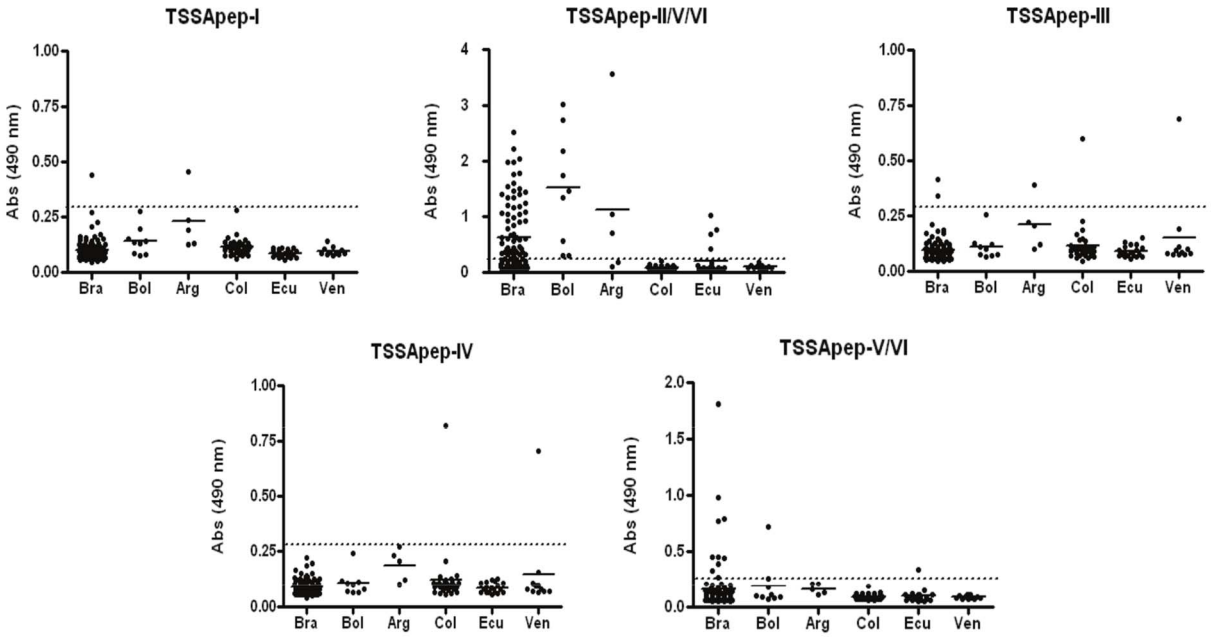

Figure 3. Recognition of TSSA lineage-specific peptides shows a disparate geographical distribution. Each data point represents the mean A_{490} of the reaction of duplicates of each serum sample with the peptides. Means of each country data set are represented by solid horizontal lines; cut-offs, derived from the mean +3SD for each peptide with the EHCs, are shown as dotted line on each graph. Circled and boxed values indicate the same sera. Resolution for the Brazilian responses to TSSApep-II/V/VI is increased by smaller symbols.
doi:10.1371/journal.pntd.0002892.g003

novel bioinformatic analysis using EpiQuest-B program that builds the immunogenicity profile for linear protein sequences and predicts the location and potential immunogenicity of the linear B-cell epitopes (Litvinov et al, in preparation) in order to give an antigenicity score for the polymorphic region. The algorithm predicted high scores within the TSSApep-II/V/VI epitope region, but much lower for TSSApep-I, as shown in Figure 4.

The different antigenicity scores indicate that the N-terminal TEN in TSSApep-II/V/VI is the dominant epitope, explaining the frequent recognition of chimera TSSApep-II/-I (Figure 1) despite the higher scoring C-terminal GEAPS, which provides a secondary site of recognition for some (Brazilian) sera that respond to chimera TSSApep-I/-II (Figure 1). Neither the TEN nor GEAPS is present in TSSApep-I, explaining the rare recognition of this epitope. The GEAPS, which is also present in TSSApep-III and TSSApep-IV, gave much lower antigenicity scores in these peptides, in the absence of the upstream TEN in these sequences (data not shown).

Comparative diversity of the putative TcI-applicable peptide reveals high conservation across lineages

Mendes et al [26] used heterozygous loci in the CL Brener genome to identify candidate lineage-specific epitopes. One conserved and three potentially lineage-specific peptides were synthesised, described as deriving from hypothetical protein Tc00.1047053510421.310 (conserved epitope peptide), putative RNA-binding protein Tc00.1047053511837.129 (for TcI serology), putative ADP-ribosylation factor GTPase activating protein Tc00.1047053511589.70 (for TcII), and putative DNA-directed RNA polymerase III subunit Tc00.1047053510359.320 (for TcVI) that displayed some discriminatory power in ELISAs and affinity-ELISAs based on differential range of absorbance values. The putative TcI epitope was described as restricted to TcI and TcVI and applicable to serological identification of a specific response to TcI. We examined the diversity in the coding sequence for this epitope, using a panel of *T. cruzi* strains across the lineages (Table 2). A single amplicon of 381bp was produced by PCR from all strains using primers MenTcI FOR and MenTcI REV. Examples from TcI and TcII strains are shown in Figure 5.

However, in comparative sequencing across isolates representing the lineages we found this epitope to be highly conserved (Table 2). This epitope had the same amino acid sequence across all the strains and lineages analysed here with the exception of strains Y and CBB (TcII) and Para 6 (TcV). In strains Y and CBB, a heterozygous nucleotide (G/C) residue led to the presence of two predicted amino acids, Ser or Thr.

The region homologous to the reported TcI-applicable epitope, which was described as specific to a TcII strain, but given only as amino acid sequence [26], was subject to BLAST against NCBI and TriTrypDB databases. There were very low stringency homologies returned by TriTryp BLAST to various *T. cruzi* proteins (mainly around the PPP tripeptide), none of which was described as RNA-binding proteins. On NCBI BLAST, highest-scoring matches were to various bacteria and fungi, none to trypanosomes. Furthermore, none of 55 sera from northern countries of South America, where TcI is highly endemic, bound to the TcI synthetic peptide reported in Mendes et al in our ELISA assays (data not shown).

Discussion

Kong et al [32] developed lineage-specific serology for the protozoan parasite *Toxoplasma gondii*, which is difficult to isolate from chronically infected patients, and most isolates of which are classified into clonal lineages type I, II, or III. Serology with synthetic peptides based on diversity within the dense granule proteins GRA6 and GRA7 was able to distinguish type II from non-type II infections in humans. Using discriminatory serology, it was possible to demonstrate that the *T. gondii* lineages had different

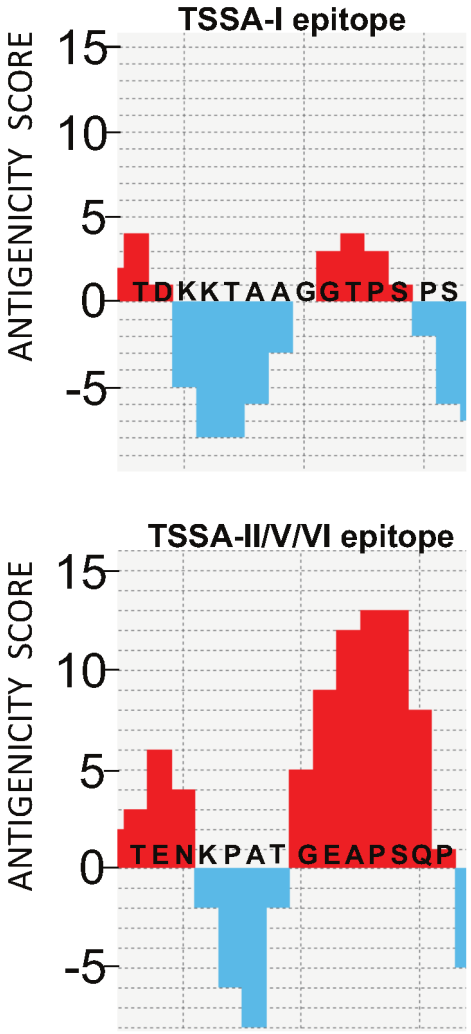

Figure 4. Computer-predicted antigenicity score is much higher for TSSA-II/V/VI sequence than TSSA-I. Polymorphic sequences of [A] TSSA-I and [B] TSSA-II/V/VI showing regions of high antigenicity in red, and low antigenicity in blue. A few amino acid replacements in TSSA-I lead to disappearance of the immunogenic epitope that is present in TSSA-II/V/VI sequence.
doi:10.1371/journal.pntd.0002892.g004

continental distributions [33], and that adult offspring of type I-infected mothers had a significantly increased risk for the development of psychoses [34].

Here we have used detailed comparative analysis of the genetic diversity of the *T. cruzi TSSA* gene encoding the protein core of the mucin TSSA, to design synthetic peptides for lineage-specific serology of *T. cruzi* infection history. These epitopes were presented on an avidin-coated solid phase via an amino terminal biotin-label linked to a polyethylene glycol-glycine spacer to increase rotation and ensure that each amino acid side chain could freely interact with antibodies. We synthesised and tested these lineage-specific peptides in ELISA with 186 human sera from six countries, three in the Southern Cone region of South America and three in northern South America. We have selected these countries because typing of *T. cruzi* isolates with multilocus enzyme electrophoresis (MLEE) [6], multilocus sequence typing (MLST) [35] and multilocus microsatellite typing (MLMT) [17] has repeatedly indicated the predominance of TcII, TcV and TcVI as the agents of Chagas disease in Southern Cone countries yet the contrasting high prevalence of TcI in patients North of the Amazon [7,11]. Nevertheless, there have been some reports that TcII, as identified by genotyping, can be found among isolates from humans and domestic triatomine bugs in northern endemic regions, for example in Colombia and Guatemala [36–38].

Lineage-specific serology is therefore of special interest for *T. cruzi*, because of the disparate geographical distributions of both the *T. cruzi* lineages and clinical manifestations of chronic Chagas disease. Thus as long ago as 1981, Miles et al proposed that the presence of chagasic cardiomyopathy with megaoesophagus and megacolon in Southern Cone countries, yet apparent absence of associated megasyndromes from Venezuela, may be related to the comparative predominance of TcI as the agent of Chagas disease in northern South America [6]. Nevertheless the evidence of a link between infecting lineage of *T. cruzi* and prognosis of chronic Chagas disease remains circumstantial. As with *T. gondii*, this is partly due to the difficulty of isolating and genotyping *T. cruzi* from chronic chagasic patients. Blood culture and xenodiagnosis have limited sensitivity and may be selective for faster growing biological clones. Furthermore, even if parasites or DNA can be recovered from chronically infected patients, the resultant *T. cruzi* isolates may not be representative of the genetic diversity in the patient, because *T. cruzi* replicates intracellularly and lineage genotypes may be sequestered in the tissues but not recoverable from the circulating blood [14]. Serology with lineage-specific antigens provides a means of profiling an individual's history of *T. cruzi* infection, to overcome inaccessibility of the parasite to direct genotyping during chronic infections.

TSSA provides a good candidate for development of synthetic peptide-based, lineage-specific serology, because no TSSA homologue beyond the species *T. cruzi* has been detected by genomic comparisons, and a lineage-specific candidate epitope can be represented by a single synthetic peptide. Thus such peptides are unlikely to generate false positive ELISA results with sera from endemic healthy controls or from patients with other infectious or autoimmune diseases. In the multiple ELISAs performed here none of the healthy controls recognised any of the synthetic peptides, and all were also serologically negative with the *T. cruzi* lysate (Figure 2). However, sera from four of the chagasic patients bound non-specifically to plates coated with avidin alone and thus

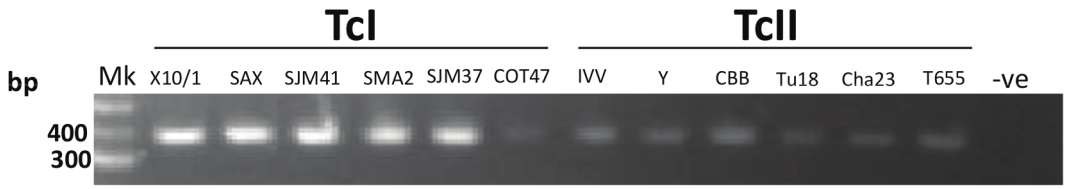

Figure 5. PCR amplification of the ORF containing the reported TcI-applicable epitope. Only the amplicon of predicted size (381 bp) was amplified by the reaction conditions. –ve = no template DNA control. Mk = Hyperladder IV (Bioline, UK).
doi:10.1371/journal.pntd.0002892.g005

Table 3. Reports of TSSA recombinant proteins in serological assays.

Reference	rTSSA Tc lineage	Assay	Sources of human sera	Authors' reports
[15]	I, II	ELISA, CL-ELISA[a]	Argentina, Brazil, Chile	TSSA dimorphism; chagasic sera only recognise rTSSA-II; TcI or TcII -infected animal sera recognised the homologous rTSSA form, without cross-reactivity.
[18]	I, II/V/VI	Western blot	Argentina	TcII/V/VI and TcI co-infection in cases of chagasic cardiomyopathy; TcII/V/VI also in indeterminate clinical form.
[19]	II	ELISA	Argentina	rTSSA-II recognised by chagasic but not non-chagasic or cutaneous leishmaniasis sera. rTSSA-II recognised by canine sera from TcVI but not TcI or TcIII infections.
[20]	II[b]	CL-ELISA [a]	Brazil	rTSSA-II 98% sensitive; no response to rTSSA-I; minimal cross-reactivity with *Leishmania* sera.
[21]	I, II	Western blot	Argentina, Colombia, Mexico, Paraguay, Venezuela	Recognition of TSSA-II, TSSA-I and TSSA-II/I in northern South America and Mexico; almost exclusively rTSSA-II in southern South America.
[22]	I, II/V/VI	Western blot, ELISA	Argentina, Bolivia, Paraguay	TcII/V/VI predominant in pregnant chagasic women; no recognition of TSSA-I reported.
[23]	II[b]	ELISA	Argentina	TcII and/or TcV/TcVI in the north of Salta province.

[a]Chemiluminescent ELISA; [b]called by authors TSSA VI, but the same as that first described as TSSA-II.
doi:10.1371/journal.pntd.0002892.t003

spuriously appeared to recognise all peptides; such artifactual binding to avidin has been observed in other serological studies [39].

Since the initial report of the sequence and antigenic dimorphism of TSSA by Di Noia et al [15], *E. coli*-produced recombinant TSSA proteins have been used as antigen with human and animal sera, as summarised in Table 3. Recognition of only TSSA-II by chronic chagasic sera from the Southern Cone region was initially interpreted as suggesting that only TcII caused chronic Chagas disease [15]. However there are many descriptions of Chagas disease and chronic chagasic cardiomyopathy in TcI endemic regions. Recognition of recombinant TSSA-I by human chagasic sera has been reported by western blot but not by ELISA [18,21]. One western blot study with recombinant TSSA-II and TSSA-I has recorded an unexpected level of TcII in northern South America and Mexico [21]. The recombinant TSSA proteins used as antigens as described encompass up to 26 amino acids flanking the polymorphic region [15,20,21], which are highly conserved between TSSA-I and TSSA-II.

The lineage-specific peptide representing the epitope common to TcII/TcV/TcVI was recognized by a large number of sera from Brazil; a proportion of these sera also bound to TSSApep-V/VI. All duplicate separate samples from the same patients gave indistinguishable results. The Brazilian sera tested here originated from the states of Goiás and Minas Gerais, where TcII human infections are known to be prevalent, TcV and TcVI are also present and TcI is (relatively) uncommon [11,40,41], although TcI is well represented among Brazilian sylvatic transmission cycles [41,42]. However, a substantial minority of the Brazilian serum samples (31/98 (31.6%)) did not react with TSSApep-II/V/VI. Thus sensitivity of the TSSApep-II/V/VI ELISA does not appear to be absolute for TcII/TcV/TcVI *T. cruzi* infections (Figure 2, Table 1). It is possible that corresponding antibodies in the TcII/TcV/TcVI seronegative patients were simply below the threshold for detection in the ELISA, although this seems unlikely because such patients remained equally seronegative against the peptides even when re-tested at the higher serum concentration of 1:100 (data not shown). Alternatively, some patients may fail to generate an immune response to the epitope or there may be as yet undiscovered TSSA diversity in some *T. cruzi* TcII strains.

Elsewhere in the Southern Cone countries 12 of 15 sera from Bolivia or Argentina were seropositive with TSSApep-II/V/VI, in accord with the known high prevalence of these lineages in those countries [23]. All sera from Bolivia, Argentina and Ecuador, and the great majority of those from Brazil, that recognised TSSApep-II/V/VI also reacted with chimera TSSApep-II/-I indicating that crucial residues reside in the N-terminal part of the TSSA-II/V/VI epitope.

We found that few serum samples from the three countries in northern South America recognized TSSApep-II/V/VI or TSSApep-V/VI. This is consistent with the literature on the geographical distribution of *T. cruzi* lineages based on genotyping of isolates from domestic and sylvatic transmission cycles. In fact only 4 sera from Ecuador were seropositive with TSSApep-II/V/VI out of 66 from these northern countries. At least 3 of these 4 Ecuadorian serum samples originated from the Loja region in southern Ecuador, where TcI has been isolated [43], close to the border with Peru. Risso et al [21] reported the identification of TcII in Colombia, Venezuela, and Mexico using western blots with TSSA-II recombinant antigen. However, when the same Colombian sera samples were tested here using the lineage-specific peptides we found no TSSApep-II/V/VI seropositive patients. Thus with our data we are unable to confirm the presence of TcII/TcV/TcVI in those Colombian patients.

Only four sera, including one from Venezuela where TcIV is known to sporadically infect humans, recognised TSSApep-IV. All four sera also recognised TSSApep-III, which shares 14 of 16 residues, presumably due to cross reaction, as we have observed with experimental murine sera (Bhattacharyya et al, in preparation).

Apart from one Argentine and one Brazilian serum, no clear specific reaction with TSSApep-I was observed, even with sera from known TcI endemic regions in Venezuela, Colombia and Ecuador. The few TSSApep-II/V/VI seropositive samples from Brazil that also reacted with chimera TSSApep-I/-II did not react with TSSApep-I. One possibility is that the TSSA-I protein, if expressed at all in chagasic patients, is not sufficiently immunogenic to generate an antibody response, possibly due to post-translational glycosylation of the core peptide sequence. Identification of the disaccharide Galα(1,3)Galβ as the immunodominant glycotope present in the O-linked mucins, i.e., those glycosylated on serine or threonine residues of the peptide chain, has been reported recently [44,45], and both serine and threonine are represented by one additional residue in TSSApep-I as compared

with the TSSA-II epitope. However, equally likely, the TSSA I epitope may be conformational, with a structure that is not represented by the linear peptide. Also, alternative immunodominant epitopes elsewhere in TSSA-I may skew the humoral response away from the sequence represented by TSSApep-I.

We were interested to see whether there was a difference in the proportions of TSSApep-II/V/VI seropositive and seronegative patients presenting with clinical symptoms of chronic Chagas disease. Remarkably, there was a clear statistically significant difference: 23/60 (38%) of the Brazilian TSSApep-II/V/VI seropositives had ECG abnormalities typical of Chagas disease, whereas such abnormalities only occurred in 4/23 (17%) of the seronegatives ($p<0.0001$). One possible interpretation of these data is that TSSApep-II/V/VI seronegative patients may not be infected with these lineages but with some less pathogenic strains. Alternatively, such seronegative patients may be infected with TcII, TcV or TcVI but the absence of an immune response to the TSSA-II/V/VI common epitope may be an indicator of a long term better prognosis; however confirmation would require a more extensive and longitudinal study. However, the frequencies of megaoesophagus (43% vs 48%) and megacolon (10% vs 8.7%) were not significantly different between the TSSApep-II/V/VI seropositive and seronegative groups respectively.

Trypanosoma rangeli is non-pathogenic to humans, is found sympatric with *T. cruzi*, particularly in northern South America, and serological cross-reaction between these species has been recognised [46]. The divergence of the TSSA epitopes in *T. cruzi* and the lack of response to the peptides with sera from northern South America, indicate that monospecific sera from patients infected with *T. rangeli* alone will not recognise these synthetic peptide epitopes.

A recent paper reported the identification of a TcI epitope for lineage-specific serology [26]. However, upon analysing the sequence diversity in the ORF coding for the parent protein across *T. cruzi* lineages, in contrast we found a very high degree of sequence conservation across the lineages. Thus, we were not able to confirm any TcI-specificity of that peptide epitope.

We have demonstrated that synthetic peptides are able to provide *T. cruzi* antigens for lineage-specific serological diagnosis in chronic Chagas disease. Synthetic-peptide based lineage-specific serology has also confirmed the disparate geographical distribution of TcII/TcV/TcVI but found fewer TcII infections in northern South America than reported with western blots and recombinant TSSA-II. Further comparisons of recombinant TSSA antigens and synthetic peptides are indicated. More attempts should be made to design a TcI specific peptide, and by comparative genomics to seek alternative antigens to TSSA that may be lineage-specific. However, such *in silico* methods will need to incorporate structural analysis, and if necessary devise linear peptides that represent conformational epitopes. In a region of Brazil endemic for TcII we find a higher rate of ECG abnormalities among patients with TSSApep-II/V/VI seropositivity than among seronegative patients. Synthetic-peptide antigens clearly have substantial and versatile potential in studying the relationship between a patient's history of infection and clinical status, and they may provide clinical biomarkers for prognosis of Chagas disease.

Author Contributions

Conceived and designed the experiments: TB AKF MAM. Performed the experiments: TB AKF TTT SVL MAM. Analyzed the data: TB AKF AOL TTT SVL MAM. Contributed reagents/materials/analysis tools: AKF AOL JAC MJG MDL LAM JDR FG HJC PD LG SVL. Wrote the paper: TB AKF AOL SVL MAM.

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PUBLICATIONS ARISING: *Leishmania donovani* complex and visceral leishmaniasis

ANNEX 3: **Bhattacharyya T**, Boelaert M, Miles MA (2013). Comparison of visceral leishmaniasis diagnostic antigens in African and Asian *Leishmania donovani* reveals extensive diversity and continent-specific polymorphisms. *PLoS Negl Trop Dis* 7 e2057

Key points, novel results and implications

- In cases of suspected visceral leishmaniasis, the rapid diagnostic test (RDT) for the presence of anti-*Leishmania* antibodies is based on the antigen rK39, a fragment of a kinesin-like protein comprising 39 amino acid repeats, originally cloned from a Brazilian *L. infantum* (syn. *L. chagasi*) strain. However recent multi-centre trials have shown much lower sensitivities in East Africa than the South Asia, for unclear reasons.
- Here, the molecular diversity of the rK39 homologous sequences among East African *L. donovani* strains was investigated as a possible factor for this differential sensitivity. Diversity of HASPB1 and HASPB2 gene repeat sequences, used to flank sequences of a kinesin homologue in the synthetic antigen rK28 designed to reduce variable RDT performance, was also investigated.
- Coding sequences of rK39 homologues from East African *L. donovani* strains were amplified from genomic DNA, analysed for diversity from the rK39 sequence, and compared to South Asian sequences. East African sequences were revealed to display significant diversity from rK39. Most coding changes in the 5' half of repeats were non-conservative, with multiple substitutions involving charge changes, whereas amino acid substitutions in the 3' half of repeats were conservative. Specific polymorphisms were found between South Asian and East African strains.
- Non-canonical combination repeat arrangements were revealed for HASPB1 and HASPB2 gene products in strains producing unpredicted size amplicons.
- This work demonstrates that there is extensive kinesin and HASPB genetic diversity among strains in East Africa and between East Africa and South Asia, with ample scope for influencing performance of diagnostic assays based on these antigens.

Candidate's contribution:

The candidate designed all the PCR primers used in this publication, following an exhaustive search of the relevant literature and databases. The candidate performed most of the cell culture, and all the DNA extractions, PCRs, gel electrophoresis, DNA sequencing preparation, alignment and analysis of the kinesin and HASPB genes as described in this publication. The candidate prepared the first draft of the manuscript, which was accepted for publication by *PLoS Negl Trop Dis* in December 2012 following academic peer review.

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Comparison of Visceral Leishmaniasis Diagnostic Antigens in African and Asian *Leishmania donovani* Reveals Extensive Diversity and Region-specific Polymorphisms

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Abstract

Background: Visceral leishmaniasis (VL), caused by infection with *Leishmania donovani* complex, remains a major public health problem in endemic regions of South Asia, East Africa, and Brazil. If untreated, symptomatic VL is usually fatal. Rapid field diagnosis relies principally on demonstration of anti-*Leishmania* antibodies in clinically suspect cases. The rK39 immunochromatographic rapid diagnostic test (RDT) is based on rK39, encoded by a fragment of a kinesin-related gene derived from a Brazilian *L. chagasi*, now recognised as *L. infantum*, originating from Europe. Despite its reliability in South Asia, the rK39 test is reported to have lower sensitivity in East Africa. A reason for this differential response may reside in the molecular diversity of the rK39 homologous sequences among East African *L. donovani* strains.

Methodology/Principal Findings: Coding sequences of rK39 homologues from East African *L. donovani* strains were amplified from genomic DNA, analysed for diversity from the rK39 sequence, and compared to South Asian sequences. East African sequences were revealed to display significant diversity from rK39. Most coding changes in the 5′ half of repeats were non-conservative, with multiple substitutions involving charge changes, whereas amino acid substitutions in the 3′ half of repeats were conservative. Specific polymorphisms were found between South Asian and East African strains. Diversity of HASPB1 and HASPB2 gene repeat sequences, used to flank sequences of a kinesin homologue in the synthetic antigen rK28 designed to reduce variable RDT performance, was also investigated. Non-canonical combination repeat arrangements were revealed for HASPB1 and HASPB2 gene products in strains producing unpredicted size amplicons.

Conclusions/Significance: We demonstrate that there is extensive kinesin genetic diversity among strains in East Africa and between East Africa and South Asia, with ample scope for influencing performance of rK39 diagnostic assays. We also show the importance of targeted comparative genomics in guiding optimisation of recombinant/synthetic diagnostic antigens.

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Editor: Philippe Büscher, Institute of Tropical Medicine, Belgium

Received September 19, 2012; Accepted December 26, 2012; Published February 28, 2013

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Funding: This work is part of the NIDIAG network research partnership supported by the European Commision under the Health Cooperation Work Programme of the 7th Framework Programme (Grant agreement no. 260200, http://cordis.europa.eu/fp7/home_en.html). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

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Leishmania promastigotes, transmitted during bloodmeal feeding by female sandflies (*Phlebotomus* and *Lutzomyia* spp., in the Old and New World respectively), are internalised by local dermal macrophages and dendritic cells. Within these host cells, flagella are lost, and transformation into proliferative amastigote forms is followed by cell lysis, re-invasion of other cells, and parasite dissemination by the lymphatic and vascular systems, which can lead to infiltration of bone marrow and hepatosplenomegaly. In symptomatic cases, VL is fatal if untreated [4].

Parasitological diagnosis, by demonstration of amastigotes in spleen aspirates, approximates to a gold standard for VL diagnosis but is applied cautiously due to associated risk. Serological (anti-*Leishmania* antibody) tests include enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT), and direct agglutination test (DAT) [5]. However, after drug treatment and cure current serological tests may still give positive results and therefore cannot readily diagnose relapse. Furthermore, such tests can also detect anti-leishmanial antibodies in asymptomatic

(Double-click on image to view embedded document)

As another possible explanation of the reported differential sensitivity of the rK39 RDT in East Africa and South Asia, the anti-*Leishmania* IgG levels of VL patients from endemic regions of Sudan and India were directly compared in ELISA against *Leishmania* lystaes originating from both regions. That work is described in the following section (ANNEX 4 Bhattacharyya *et al*., 2014).

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Editor: Philippe Büscher, Institute of Tropical Medicine, Belgium

Received September 19, 2012; **Accepted** December 28, 2012; **Published** February 28, 2013

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Funding: This work is part of the NIDIAG network research partnership supported by the European Commission under the Health Cooperation Work Programme of the 7th Framework Programme (Grant agreement no. 260260, http://cordis.europa.eu/fp7/home_en.html). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Author Summary

Visceral leishmaniasis (VL) is caused by infection with parasites of the *Leishmania donovani* complex, spread by the bite of blood-sucking sandflies, especially in South Asia, East Africa, and Brazil. If untreated, symptomatic VL leads to systemic pathologies and is usually fatal. Up to approximately 400,000 new cases are estimated to occur annually, and regional epidemics have been devastatingly severe. Diagnosis of clinically suspect cases in the field, and thus appropriate treatment, relies principally on a rapid diagnostic test (RDT) based on detection of antibodies against a *Leishmania* antigen known as rK39. Although this test is reliable in South Asia, it has shown less success in East Africa. One reason may reside in diversity of the rK39 homologue among East African *L. donovani*, with consequent limitations in the binding of diagnostic antibodies. Here, we investigate the sequences of rK39 homologues in a panel of East African *L. donovani* strains, and show that there is significant diversity compared to rK39 and to South Asian sequences. Additionally, we examine diversity in another diagnostic antigen known as HASPB1/2. Our results indicate that an improved RDT may need to encompass East-African-specific antigen diversity to provide high performance field diagnosis for this region.

individuals living in endemic areas, but with no VL history or subsequent progression to VL [6].

Burns et al [7] identified a kinesin-related gene product, LcKin, as a candidate diagnostic antigen by screening a Brazilian *L. infantum* (*L. chagasi*) genomic library with serum of an *L. donovani* patient. A part of the coding sequence, comprising a 46aa region followed by 6.5×39aa repeats, forms the recombinant diagnostic protein rK39. In recent multicentre evaluations, the use of the rK39 in a lateral-flow immunochromatographic, rapid test format reported less success in East Africa than in the Indian subcontinent for point-of-care diagnosis of VL [8,9]. Underlying explanatory factors may reside in molecular divergence between East African *L. donovani* kinesin gene homologues and the Brazilian *L. infantum* (*L. chagasi*)-derived rK39 sequence, and/or may be due to differential immunocompetence and antibody levels produced among African and Asian human populations.

Studies on South Asian *L. donovani* strains using PCR primers based on *LcKin* have identified rK39 homologous sequences [10–12]. Gerald et al [13] reported the first East African (Sudanese) *L. donovani* kinesin homologue, LdK39. The first two of the 39-aa repeats of LdK39, flanked by sequences of the *L. donovani* antigens HASPB1 and HASPB2 [14], comprise rK28, a novel recombinant protein for diagnosis of VL, designed to be an improvement over rK39 [15–16]. HASPB proteins are expressed on the surface of infective promastigote and amastigote *Leishmania* life cycle stages [14]. The first 3×14aa repeats of HASPB1 are incorporated into rK28, along with the complete ORF of HASPB2, which includes three imperfect consecutive repeats, 2×14aa and 1×13aa. HASPB1 and HASPB2 correspond with K26 and K9, respectively [17], which were originally identified in *L. infantum* (*L. chagasi*) at the same time as the HASPBs, but were given different nomenclature. A recent comparison of rK26 and rK9 showed significantly lower diagnostic efficacy than rK28 [15].

Here we investigate whether molecular divergence in rK39 kinesin sequence homologues of East African *L. donovani* may contribute to lower rK39 diagnostic test success rates in East Africa. We analyse the rK39 homologues in a panel of East African *L. donovani* strains and compare their diversity against published South Asian sequences. In addition, we compare sequence diversity of HASPB1 and HASPB2 in East African and South Asian strains.

Methods

L. donovani strains

Table 1 lists the East African *L. donovani* strains used in this study. Strains were selected to represent genetic groups within the *L. donovani* complex in East Africa (Baleela et al, unpublished data; identified by multilocus sequence typing (MLST) and microsatellite analysis (MLMT)). Strains were cultured in αMEM medium supplemented by foetal calf serum (Sigma, UK), and genomic DNA was extracted from uncloned cultures using Gentra Puregene Tissue Core Kit A (Qiagen, UK). Uncloned cultures were specifically used here because the intention was to capture the diversity present within natural *L. donovani* populations. Table 2 lists the GenBank sequences derived in other studies [7,10–14,18–19] and used here for comparisons.

Kinesin homologues

PCR primers LdonK39F (gagctcgcaaccgagt) and LdonK39R (ctgrctcgccagctcc) were designed for this study based on a comparison of LcKin and LdK39 coding sequences (Figure 1A), and were targeted to amplify the 894 bp region of the *L. donovani* kinesin gene that is incorporated into the diagnostic antigen rK39. The level of conservation in the sequences encoding the 39-aa repeats predicts that primer LdonK39R would be expected to anneal to multiple sites within the kinesin gene template, generating multiple amplicons. Amplification reactions were performed in a total volume of 20 ul, and comprised of 1× NH_4 Reaction buffer supplemented with 1.5 mM $MgCl_2$ (Bioline, UK), 200 μM dNTPs (New England Biolabs, UK), 10 pmol of each primer, and 1 U BioTaq DNA polymerase (Bioline). Amplification conditions were: 1 cycle of 94°C, 2 mins; 25 cycles of 94°C for 30 secs, 55°C for 30 secs, 72°C for 1 min; 1 cycle of 72°C for 5 mins. PCR products were separated by electrophoresis on 1.5% agarose gels (Bioline). Amplicons corresponding to rK39 homologues were excised and purified from gels using QIAquick Gel Extraction Kit (Qiagen), cloned into pGEM-T easy vector (Promega, UK), and transformed into XL1-blue *E. coli* on blue-white selection. Primers Sp6/T7 and M13for/rev were used to sequence from the vector (between 2–5 colonies per strain were taken forward for sequencing, except UGX-MARROW and SUDAN1, where due to difficulty in cloning the corresponding inserts only one clone was sequenced); in addition, LdonK39int (cgagcggctaaccagc), which binds to the 3′ end of the non-repeat region immediately upstream of the repeats, was used as an internal sequencing primer (Figure 1A). Sequences were analysed using BioEdit [20].

HASPB

PCR primers LdonHASPBfor (cataaaaccactgaggc) and LdonHASPBrev (atcttcgttcttctcctg) were designed for this study to flank the repeat regions of the HASPB1 ORF, amplifying a 1064 bp product (Figure 1B). Due to the identity between HASPB1 and HASPB2 at the primer binding sites, a 260 bp product would also be predicted to be amplified from HASPB2 by these primers (Figure 1C). Composition of PCR mix was as described above for kinesin, except that 40 μM dNTPs were used. Amplification conditions were: 1 cycle of 94°C, 2 mins; 25 cycles of 94°C for 30 secs, 50°C for 30 secs, 72°C for 1 min; 1 cycle of 72°C for 10 mins. PCR products were separated by electrophoresis on 1% agarose gels (Bioline). Amplicons were excised and purified from

Table 1. East African *L. donovani* strains for which sequences were determined, with GenBank accession numbers.

Strain	Origin	MON/LON[a]	Kinesin	HASPB amplicon		
				HASPB1	HASPB2	Unpredicted
MHOM/ET/67/HU3 (LV9)	Ethiopia	MON18/LON46	KC342866	KC342849	KC342855	-
MHOM/ET/00/HUSSEN	Ethiopia	MON31/LON42	KC342867	-	-	KC342861
MHOM/ET/72/GEBRE1	Ethiopia	MON82/LON50	-	KC342850	-	-
MHOM/SD/87/UGX-MARROW	Sudan	MON31	KC342868	-	-	KC342862
MHOM/SD/82/GILANI	Sudan	MON30/LON48	KC342869	KC342851	KC342856	-
MHOM/SD/98/LEM3582	Sudan	MON18	KC342870	KC342852	KC342857	-
MHOM/SD/XX/SUDAN1	Sudan	MON18	KC342871	-	KC342858	-
MCAN/SD/98/LEM3556	Sudan	MON82	-	KC342853	KC342859	-
MHOM/SD/97/LEM3458	Sudan	MON18	-	KC342854	KC342860	-
IMAR/KE/62/LRC-L57	Kenya	MON37/LON44	KC342872	-	-	KC342863
MHOM/KE/67/MRC(L)3	Kenya	MON37/LON44	-	-	-	KC342864
MCAN/IQ/81/SUKKAR 2	Iraq	LON43	-	-	-	KC342865

[a]MON and LON reference numbers refer to multilocus sequence enzyme electrophoresis (MLEE) profiles.
doi:10.1371/journal.pntd.0002057.t001

gels using QIAquick Gel Extraction Kit, and sequenced directly using the amplification primers. Sequences were analysed using BioEdit.

Accession numbers

Kinesin nucleotide sequences derived in this manuscript are available under GenBank accession numbers KC342866-KC342872. HASPB Nucleotide sequences derived in this manuscript are available under GenBank accession numbers KC342849- KC342865.

Results

East African *L. donovani* kinesin diversity

Multiple kinesin amplicons were produced using a combination of primers LdonK39F, which binds to the non-repeat region and LdonK39R, because the latter primer binds to nucleotide sequence that is conserved across the repeats. An example is shown in Figure 2. Sequencing of cloned amplicons containing the rK39 homologous sequences into plasmid vectors revealed the presence of nucleotide and predicted amino acid diversity among East African *L. donovani* strains. Table 3 shows the amino acid polymorphisms that were found among the East African strains, together with their divergence from *L. infantum* (*L. chagasi*) derived LcKin rK39, and alongside the GenBank sequence for *L. donovani* derived kinesin LdK39 used in rK28. Substitutions between a non-charged and a charged residue (D^-, E^-, H^+, K^+, R^+) in comparison with the LcKin rK39 sequence are shown underlined.

When the seven rK39 homologous repeats of all the East African strains were compared with the LcKin rK39 amino acid sequence, residues 2, 6, 10, 16 and 18 were each affected three or four times by substitutions involving charge changes. In contrast, although there were multiple substitutions in the latter half of the East African rK39 tandem repeats, especially affecting residues 21,

Table 2. GenBank sequences used in comparisons.

Strain	Origin	Gene product	GenBank	Reference
MHOM/BR/82/BA-2,C1	Brazil	Kinesin LcKin/rK39	L07879	[7]
MHOM/IN/KE16/1998	India	Kinesin/Ld-rKE16	AY615886	[10]
Morena[a]	India	Kinesin	DQ648599	[11]
MHOM/IN/80/DD8	[b]	Kinesin/rKRP42	AB256033	[12]
MHOM/SD/62/1S-CL2D	Sudan	Kinesin/LdK39	DQ831678	[13]
MCAN/ES/98/LLM-877 (JPCM5)[c]	Spain	Kinesin	XM_00146426[e]	[18]
MHOM/NP/2003/BPK282/0cl4[d]	Nepal	Kinesin	FR799601[e]	[19]
MHOM/ET/67/L28 (LV9)	Ethiopia	HASPB1	AJ011810	[14]
MHOM/ET/67/L28 (LV9)	Ethiopia	HASPB2	AJ011809	[14]
MHOM/NP/2003/BPK282/0cl4[d]	Nepal	HASPB	FR799601[e]	[19]

[a]Described in [11] as clinical isolate, not laboratory strain, so WHO code not given here.
[b]Described in [12] as isolated from a Bangladeshi patient, but MHOM/IN/80/DD8 is also reported as being from India [6].
[c]*L. infantum* reference genome.
[d]*L. donovani* reference genome.
[e]GenBank numbers refer to locations on the entire chromosome 23 sequence, to which both the kinesin and HASPB BLAST searches map.
doi:10.1371/journal.pntd.0002057.t002

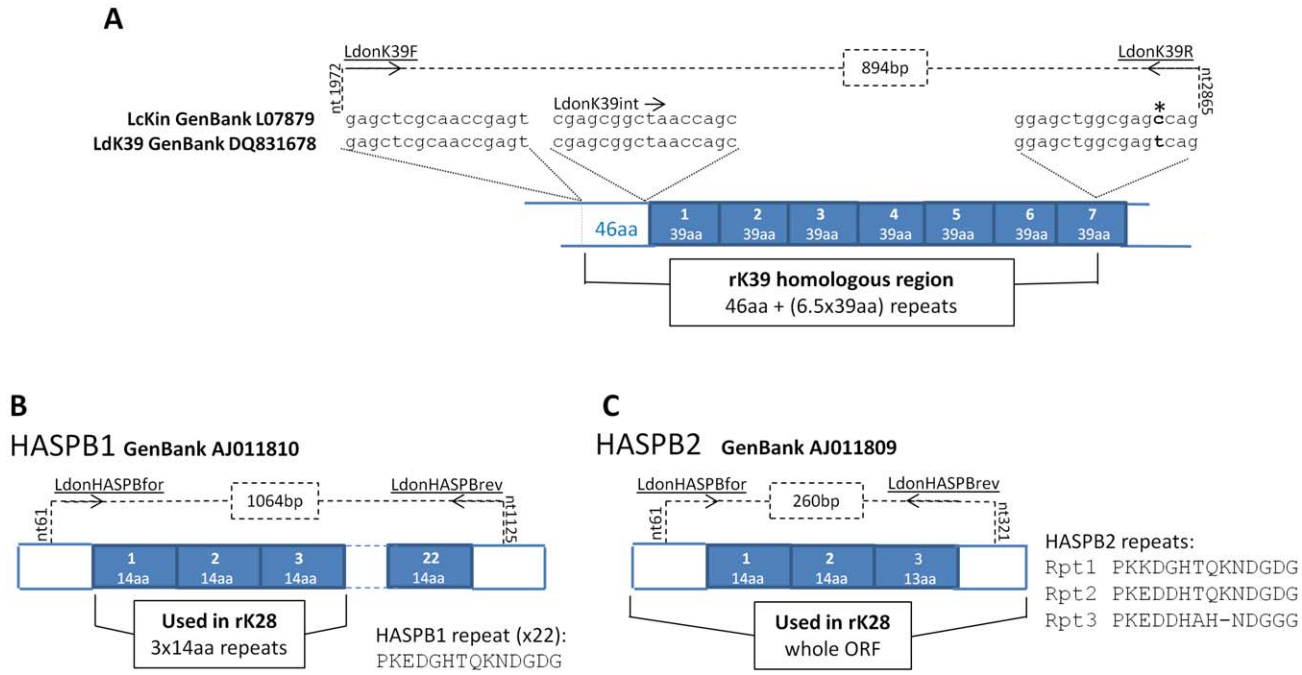

Figure 1. *Leishmania* rK39 and HASPB antigen repeats and the PCR primer target sequences. Repetitive coding regions depicted as filled boxes, PCR primers underlined, and 5′ and 3′ binding positions with amplicons are indicated by dashed lines. [A] Kinesin gene comparison for primer design (* = non-conserved nucleotide). [B] HASPB1 GenBank sequence displays 22× perfect 14aa repeats. [C] HASPB2 GenBank sequence displays 3 imperfect repeats.
doi:10.1371/journal.pntd.0002057.g001

23, 27 and 39, none of these substitutions involved charge changes. Two tracts of the rK39 repeat, residues 11 to 15 and 28 to 34 were perfectly conserved between LcKin39 across all the East African strains examined and across all seven rK39 repeats within those strains.

Sequencing of multiple plasmid clones revealed the presence of alternative residues at single sites within single strains. This was notably widespread for the two strains Gilani and Hussen, in the case of Gilani, including several alternatives within the first and second repeats.

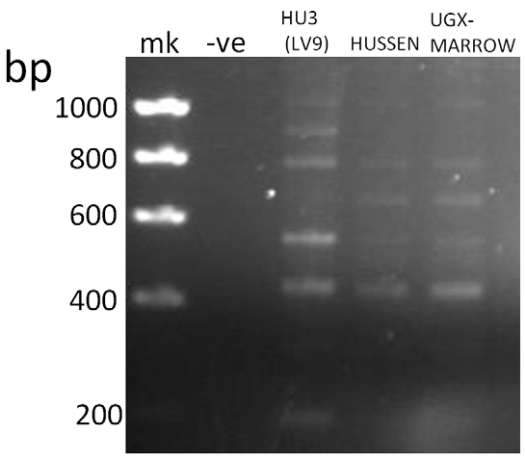

Figure 2. Multiple amplicons corresponding to kinesin tandem repeats are produced by PCR primers LdonK39F and LdonK39R. Amplifications from strains HU3 (LV9), Hussen, and UGX-MARROW, are depicted. Major amplicon sizes differ by 117 bp, the size of the nucleotide sequence encoding the 39aa repeat in the kinesin gene; mk = Hyperladder I (Bioline).
doi:10.1371/journal.pntd.0002057.g002

Comparison of *L. donovani* kinesin diversity between East Africa and South Asia

Figure 3 depicts the composite *L. donovani* kinesin polymorphisms for East Africa and South Asia and the divergence between the two geographic regions, in comparison with the LcKin rK39 derived from *L. infantum* (*L. chagasi*). The East African polymorphisms in Figure 3 incorporate the data obtained here together with the published LdKin (rK28) sequence. Substitutions between non-charged and charged residues (D^-, E^-, H^+, K^+, R^+) compared to the LcKin rK39 sequence are shown underlined. East Africa-specific polymorphisms are boxed; South Asia-specific polymorphisms are circled.

In the 46aa non-repeat region the only divergence from *L. infantum* (*L. chagasi*) diagnostic LcKin rK39 was Cys→Ser^{41}, found among both South Asian and East African strains (Table 3). Throughout the 6.5×39aa repeats, there were polymorphisms divergent from the diagnostic LcKin rK39 that were unique to each region and other polymorphisms that were common to East Africa and South Asia. For example, Glu→Gly^{2} (Repeats 4, 5, 7) and Arg→His^{11} (Repeats 1, 2, 4) are found only in East Africa and South Asia respectively, whereas in Repeat 1, Ala→Ser^{21}, Ala→Thr^{23}, Ser→Met^{27}, Thr→Ala^{39}, which are all conservative changes found in the latter half of the repeats, were found in both regions. Indeed, as for the East African strains (Table 3) all such polymorphisms identified in the latter half of the repeats among South Asian strains are conservative, *i.e.*, a non-charged residue is replaced by another non-charged residue; conversely, non-conservative substitutions between non-charged and charged residues are found in the first half of the repeats. Among the published South Asian sequences charges diverged from those in

Table 3. rK39 polymorphism among East African *L. donovani* strains and in comparison with the LcKin diagnostic rK39 sequence.

	P[c]	Rpt1					Rpt2								Rpt3							
Amino Acid	41	4	21	23	27	39	6	7	10	21	23	24	27	39	7	8	10	21	23	27	35	39
LcKin (used in rK39)[a]	C	Q	A	A	S	T	R	E	A	A	A	A	M	T	D	S	E	S	T	M	S	T
LdK39 (used in rK28)[b]	S	L	S	T	M	T	R	D	E	A	A	A	S	A	D	S	E	S	T	T	N	T
MHOM/ET/67/HU3 (LV9)	S	L	S	T	M	T	R	D	E	A	A	A	S	A	D	S/P	E	S	T	T	N	T
MHOM/ET/00/HUSSEN	S	Q	A	A	S	A	R	E	E	A	A	A	S/P	A	E	S	A	S/A	T/A	M/S	N	T
MHOM/SD/87/UGX-MAR	S	Q	A	A	S	A	R	E	E	A	A	A	S	A	E	S	A	S	T	M	N	T
MHOM/SD/82/GILANI	S	L	S/A	T/A	M/S	T	R	D	E	S/A	T/A	A	M/S	T/A	D	S	E	A/S	A/T	S/T	N	A/T
MHOM/SD/98/LEM3582	S	L	S	T	M	T	R	D	E	A	A	V/A	S	A	D	S	E	S	T	T	N	T
MHOM/SD/XX/SUDAN1	S	L	S	T	M	T	R	D	E	A	A	A	S	A	D	S	E	S	T	T	N	T
IMAR/KE/62/LRC-L57	S	Q	A	A	S	T	L	E	E	S	T	A	M	A	E	S	E	A	A	S	N	T

	Rpt4									Rpt5										
Amino Acid	2	6	7	10	21	23	27	35	39	2	6	7	16	17	18	21	23	27	35	39
LcKin (used in rK39)	E	R	D	E	S	T	M	S	T	E	R	E	A	S	Q	S	T	M	S	T
LdK39 (used in rK28)	E	R	D	E	S	T	M	N	A	E	L	E	K	A	E	A	A	S	N	A
MHOM/ET/67/HU3 (LV9)	E	R	D	E	S	T	M	N	A	E	L	E	K	A	E	A/V	A	S	N	A
MHOM/ET/00/HUSSEN	E/G	R	E	E/A	A	A/T	S/M	N	T	G/E	R	E	A	S	Q	A	T/A	S	N	A
MHOM/SD/87/UGX-MAR	G	R	E	A	A	A	S	N	T	G	R	E	A	S	Q	A	T	S	N	A
MHOM/SD/82/GILANI	E	R	D	E	S	T	T/M	N	T/A	E	R/L	D/E	A/K	S/A	Q/E	S/A	T/A	M/S	N	A
MHOM/SD/98/LEM3582	E	R	D	E	S	T	M	N	A	E	L	E	K	A	E	A	A	S	N	A
MHOM/SD/XX/SUDAN1	E	R	D	E	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-
IMAR/KE/62/LRC-L57	E	L	E	E	S	T	M	N	A	E	R	E	A	S	Q	A	A	S	N	A

	Rpt6									Rpt7					
Amino Acid	6	7	16	17	18	21	23	27	39	2	7	10	16	17	18
LcKin (used in rK39)	R	D	A	S	Q	A	A	S	A	E	D	E	A	S	Q
LdK39 (used in rK28)	R	D	A	S	Q	S	T	T	T	E	D	E	A	S	Q
MHOM/ET/67/HU3 (LV9)	R	D	A	S	Q	S	T	T	T	E	D	E	A	S	Q
MHOM/ET/00/HUSSEN	R	E	A	S	Q	S/A	T/A	M/S	A/T	E/G	E	E/A	A/K	S/A	Q/E
MHOM/SD/87/UGX-MAR	R	E	A	S	Q	A	A	S	T	G	E	A	K	A	E
MHOM/SD/82/GILANI	L/R	E/D	K/A	A/S	E/Q	A	A/T	S	A	E	D	E	A	S	Q
MHOM/SD/98/LEM3582	R	D	A	S	Q	S	T	T	T	E	D	E	A	S	Q

Table 3. Cont.

	Rpt6							Rpt7						
Amino Acid	6	7	16	17	18	21	23	27	39	2	7	10	16	17	18
MHOM/SD/XX/SUDAN1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IMAR/KE/62/LRC-L57	$^{H}_{R}$	E	A	S	Q	A	A	S	A	E	E	E	A	S	Q

[a]LcKin is derived from *L. infantum* (*L. chagasi*).
[b]LdK39 is derived from *L. donovani*.
[c]P = pre-repeat region.
– = not determined here.
doi:10.1371/journal.pntd.0002057.t003

diagnostic LcKin rK39 at residue 4 (Gln→Arg; Repeats 2, 3, 4) and at residues 16, 17 and 18, for example uncharged to positive at these three sites in repeat 1 (Figure 3).

Comparison with *L. donovani* complex reference genomes

After the sequencing of the *L. infantum* reference genome [18], Downing et al [19] sequenced the Nepalese *L. donovani* strain BPK282/0cl4 as the reference genome for *L. donovani*. The sequence coding for the non-repeat 46-aa region of rK39 was submitted to NCBI BLAST against the reference genomes. For the *L. infantum* reference genome (Spanish, canine-isolated), there were no differences across the entire rK39 sequence, in accordance with this being the postulated source of *L. infantum* (*L. chagasi*) in South America [3]. For the genome sequence of BPK282, due to the repeat nature of the downstream region, only sequences of the first two repeats could be unequivocally aligned and these were incorporated into Figure 3 for comparisons with the individual repeat sequences generated here. From the unequivocal BLAST alignment information, BPK282 repeat 1 is the same as for rK39 except Thr→Ala[39]; in BPK repeat 2, where only the first 6 residues are unambiguously assembled, Gln→Arg[4] is present.

L. donovani HASPB

PCR primers LdonHASPBfor and LdonHASPBrev were designed to bind unique sequences flanking the 22×14aa-repeat coding region of HASPB1 to produce a 1064 bp amplicon. However, with some of the strains studied here in addition to the 1064 bp product the smaller 260 bp amplicon corresponding to HASPB2 (Figure 1C) could also be seen: an example is shown for HU3 (LV9) in Figure 4. However, these primers unexpectedly gave amplicons of ~400–500 bp for some of the strains (Hussen, UGX-marrow, LRC-L57, MRC(L)3, SUKKAR 2) (Figure 4).

Comparisons with rK28 HASPB sequences

Strains HU3 (LV9), Gebre1, Gilani, LEM3582, LEM3556, and LEM3458, which gave 1064 bp-amplicons, had identical sequences in their first 3×14aa repeats as the HASPB1 used in diagnostic rK28. However, subsequent repeats, not incorporated into rK28, were imperfect with polymorphic residues at certain sites, such as His→Arg[6] (repeats 15, 18, 22) and Gly→Ala[14] (repeats 5, 14, 17) for strains LEM3582, LEM3556, and LV9.

For those strains producing the predicted 260 bp amplicon of the HASPB2 homologue, (Figure 1) a comparison with the rK28 sequence identified a Gly residue inserted between Ala[32] and Val[33] preceding the imperfectly repeated 14/13aa region and a Pro→Gln[96] substitution present after the 14/13aa region. The only polymorphism within the imperfectly repeated 14/13aa region, compared to rK28, was Lys→Glu[49], at the third residue of first 14aa region, with a consequent charge change.

Interestingly, the strains producing unexpected ~400–500 bp amplicons (Hussen, UGX-marrow, LRC-57, SUKKAR 2, MRC(L)3) revealed on sequence analysis a HASPB1/HASPB2-like combination composition across their repeats, due to the presence of the central motifs that were HASPB1-like (HTQKN) or HASPB2-like (HAH-N). The HTQKN and HAH-N motifs were not consistently present in the same repeat numbers across these five strains and the HASPB1-like and HASPB2-like sections of the tandem repeat were not necessarily aggregated (Table 4). In the case of LRC/L57, both the 1064 bp (predicted HASPB1-homologue) and ~500 bp amplicons contained the HASPB2-like motif HAH-N. The Lys→Glu[49] substitution at the third residue of

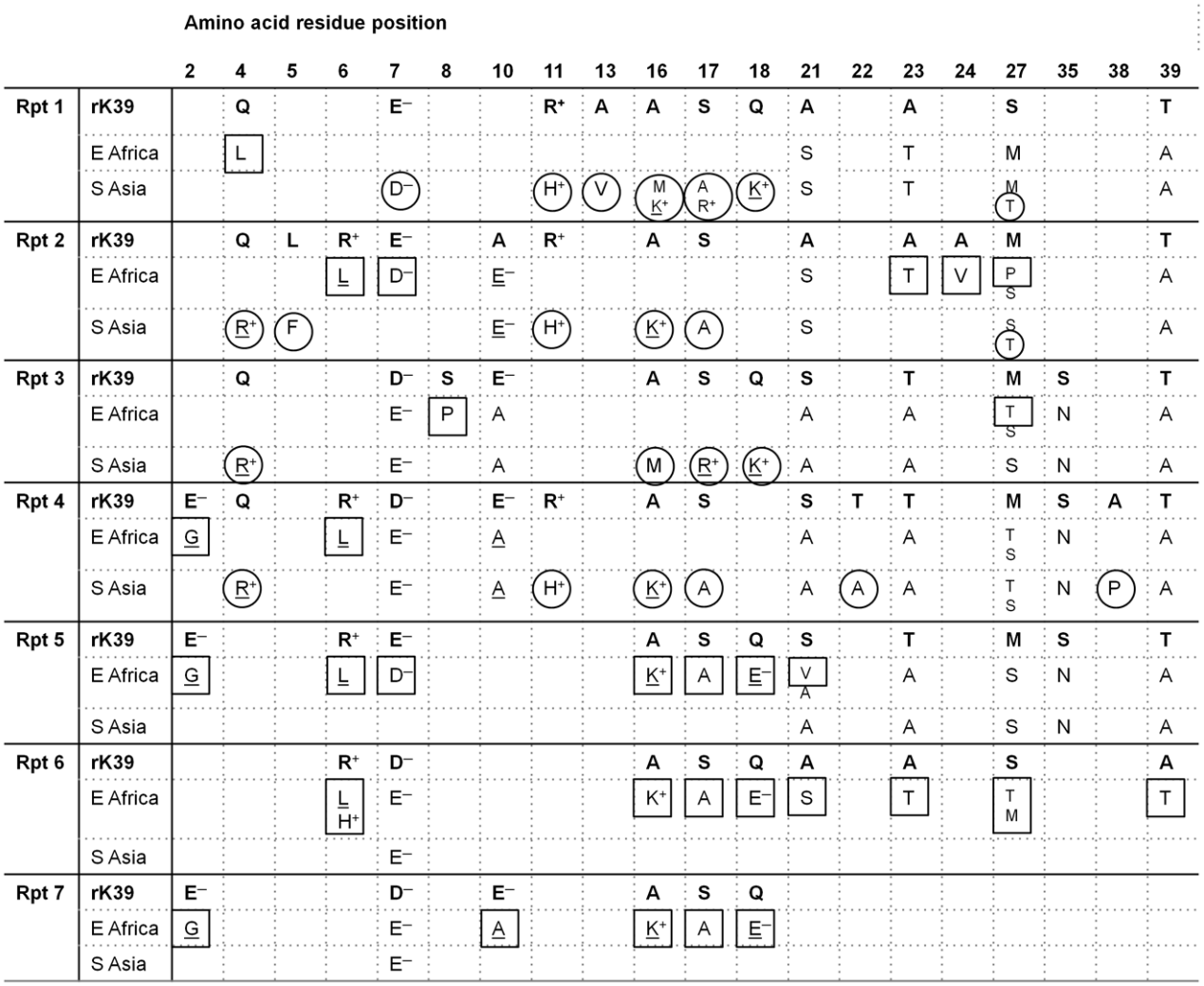

Figure 3. Polymorphisms among seven rK39 repeats of East African and South Asian strains show region-specific substitutions. Changes between charged and non-charged residues are underlined. No residue entered at a particular site indicates conservation of that residue with the corresponding residue of diagnostic rK39. Where two alternative residues are indicated in smaller text these are not necessarily always within the same strain, for clarification compare with Table 3. Region-specific polymorphisms in each repeat are boxed (East Africa) or circled (South Asia).
doi:10.1371/journal.pntd.0002057.g003

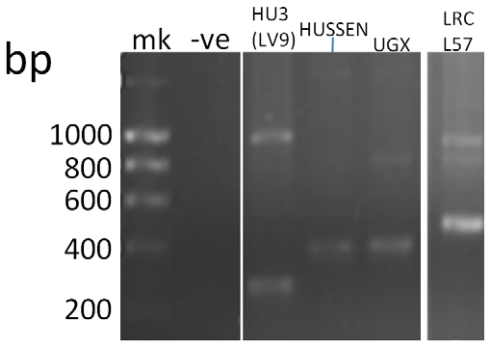

Figure 4. Predicted (1064 bp, 260 bp) amplicons and unpredicted (~400–500 bp) amplicons with HASPB PCR primers LdonHASPBfor and LdonHASPBrev. Amplifications from strains HU3 (LV9), Hussen, UGX-MARROW, and LRC-L57, are depicted; mk = Hyperladder I.
doi:10.1371/journal.pntd.0002057.g004

the first 14aa region, described above, was also present in those strains giving the ~400–500 bp amplicons.

Comparison with *L. donovani* reference genome

The sequence coding for the pre-repeat region of HASPB1 (not used in rK28, Figure 1A) was submitted to NCBI BLAST against the Nepalese *L. donovani* reference genome, to facilitate correct alignment. Due to the repeat nature of the downstream region, only the first two repeats of the reference genome could be unequivocally aligned for comparisons with the individual sequences generated here. The first two repeats of the reference genome showed the combination composition of central motifs HTQKN in repeat 1 (HASPB1-like) and HAH-N in repeat 2 (HASPB2-like). Thus, the South Asian reference genome has a HASPB1/2 combination structure like the four East African strains Hussen, UGX-marrow, LRC-57, MRC(L)3, and the Iraqi SUKKAR 2, and with the same organisation of repeats 1 and 2 seen in the Kenyan strains LRC-57 and MRC(L)3.

Table 4. Repeat sequences of unexpected amplicons.

	HUSSEN	UGX-Marrow	LRC-L57	MRC(L)3	SUKKAR 2
Rpt 1	LKEDGHTQKNDGDG	LKEDGHTQKNDGDG	PKEDGHTQKNDGDG	PKEDGHTQKNDGGG	LKEDGHTQKNDGDG
Rpt 2	PKEDGHTQKNDGGA	PKEDGHTQKNDGGA	PKEDDHAH-NDGGG	PKEDDHAH-NDGGG	PKEDGHTQKNDGDG
Rpt 3	PKEDGHTQKNDGDG	PKEDGHTQKNDGDG	PKEDDHAH-NDGGG	PKEDDHAH-NDGGG	PKEDDHAH-NDGDG
Rpt 4	PKEDDHAH-NDGDG	PKEDDHAH-NDGDG	PKEDGHTQKNDGDG	PKEDGHTQKNDGDG	PKEDDHAH-NDGDG
Rpt 5	PKEDDHAH-NDGGG	PKEDDHAH-NDGGG	PKEDDHAH-NDGGG	PKEDDHAH-NDGGG	PKEDDHAH-NDGGG
Rpt 6	PKEDDHAH-NDGGA	PKEDDHAH-NDGGG	PKEDDHAH-NDGGG	PKEDDHAH-NDGGG	PKEDDHAH-NDGGG
Rpt 7			PKEDDHAH-NDGGG	PKEDGHTQKNDGGV	
Rpt 8			PKEDGHTQKNDGGV		

Central motifs:
HTQKN = HASPB1-like.
HAH-N = HASPB2 like.
doi:10.1371/journal.pntd.0002057.t004

Discussion

For VL there is a particular need for rapid diagnostic tests (RDTs) that can be unequivocally applied in endemic regions at primary care level and with only basic technical training. Of the available options for serological diagnosis of VL only the immunochromatographic rK39 assay can be considered a point of care test for field application. The IFAT requires a fluorescence microscope. Use of the DAT is constrained by the readout delay, the relative sophistication of the procedure and limited access to lyophilised antigen. In agreement with other studies, Boelaert et al [8] recommend the use of rK39 in South Asia, but report less satisfactory results from East Africa. One factor influencing regional differences in diagnostic performance may be divergence in kinesin gene homologues in East African *L. donovani* from the *L. infantum* (*L. chagasi*)-derived LcKin rK39 sequence. In an attempt to overcome the geographical limitations of the rK39 test a modified recombinant antigen, rK28, has been devised, incorporating segments of the HASPB antigen.

Analyses of *L. donovani* complex isolates with multiple molecular markers have revealed extensive genetic diversity with at least six distinct lineages, and both inter- and intra-lineage diversity. Furthermore, genotyping of isolates from endemic regions has shown an association between genotype and geographical origin [21–22]. Kuhls et al [22] used multilocus microsatellite typing (MLMT) for high resolution comparison of the six global *L. donovani* genetic lineages, and showed that allelic diversity in Africa was greater than in India; a subsequent MLMT study also described comparative homogeneity among South Asian *L. donovani* [23]. Thus, the relative homogeneity of South Asian *L. donovani* and greater diversity in East Africa, if mirrored in diversity of the kinesin gene, is one potential explanation of the more limited efficacy of diagnostic rK39 in East Africa.

Here we determined across a panel of East African strains the diversity of the kinesin repeat region homologous to that used in both the rK39 diagnostic antigen (repeats 1 to 6.5) and the rK28 recombinant antigen (repeats 1 and 2). We then compared this diversity to that among predetermined South Asian sequences.

Surprisingly, in view of the proven value of the rK39 RDT in South Asia, there is considerable amino acid diversity among kinesin repeats 1 to 7 of South Asian strains as well as those from East Africa (Figure 3). Alternative residues at multiple sites suggested that Gilani and Hussen are heterozygous, although sequences were not obtained from DNA clones and therefore multiclonality rather than heterozygosity cannot be excluded. Across all strains the diversity was particularly notable in the first half of each rK39 repeat, because it involved charge changes at residues 2, 6, 10, 16, and 18, with only a short stretch of consistently conserved residues (11 to 15). Such charge changes among the South Asian strains are very likely to disrupt antigenic epitopes, if present. As far as we are aware epitopes within or across adjacent rK39 repeats have not yet been precisely mapped. However, we would predict that the diagnostic epitopes lie in the latter half of the rK39 repeat, where, although there is extensive diversity, the changes are conservative, none involving charge, and there is one stretch of residues (28 to 34) that is entirely conserved across all isolates and across all seven repeats. This is consistent with the recent work of Costa et al [24] in which antigen prediction software led to the synthesis of a 22mer peptide (ESTTAAKMSAEQDRESTRATLE) encompassing the region of rK39 from residue 20 of repeat 3 to residue of 2 of repeat 4, a sequence which is also found in the next two repeats. In that study, the peptide was recognised in ELISA by Brazilian sera from symptomatic and asymptomatic canine VL and symptomatic human VL. However, our study shows that at least 5 of the residues within that 22mer are polymorphic in East Africa.

Kinesin repeat divergence from the rK39 diagnostic recombinant is complex, and some divergence is common to both South Asia and East Africa, for example at residues 7, 10, 21, 23 (Figure 3). Nevertheless, there were polymorphisms unique to each region, for example at residues 4, 16, and 18, consistent with the expectation that differential sensitivities of rK39 assays may be partially attributable to positional and physiochemical polymorphisms of the kinesin gene. The identification and confirmation of region-specific polymorphisms may be limited by the amount of sequence data so far available. Also diversity beyond the seven sequenced repeats of the kinesin tandem array may present a wider range of epitopes for immunological recognition.

The differential immunocompetence and antibody titres of East African and South Asian human populations in the context of VL remains to be thoroughly explored, as a major alternative or contributing explanation of variable rK39 test sensitivities. Interestingly a recent WHO/TDR evaluation [9] of a rapid diagnostic test based on rKE16 antigen, a LcKin homologue derived from an Indian *L. donovani* [10], reported markedly lower sensitivities against Brazilian and East African sera, compared to South Asian. The same evaluation [9] also reported a lower sensitivity of rK39-based tests with Brazilian sera. However, the

rK39 antigen is derived from a Brazilian *L. infantum* (*L. chagasi*) strain [7], which originated from Iberia in Europe and is considered to be relatively homogeneous, based on microsatellite typing [3]. Thus, kinesin diversity among Brazilian *L. infantum* should be explored further and comparative immune response levels among Brazilian and South American populations.

In the rK28 recombinant diagnostic antigen, designed to improve on performance of the rK39 test, highly conserved HASPB repetitive amino acid sequences flank the first two repeats of Ldk39, derived from an East African strain. Alce et al [14], following the work of McKean et al [25], amplified two homologues of the *L. major* Gene B protein from *L. donovani*, naming the ORF of the larger HASPB1 and that of the smaller HASPB2. Recombinant HASPB1 has been used in animal vaccine models [26–27], and HASPB has a role in *L. major* differentiation within the sandfly vector *P. papatasi* [28]. The PCR primers used in the current study, like those used by Alce et al [14], anneal to regions flanking HASPB repeat regions and generate two PCR products of predicted size corresponding to HASPB1 and HASPB2, compatible with the presence of two distinct HASPB1 and HASPB2 loci in the genome, as concluded for the *L. major* genome [29]. However, several strains produced unexpected sized amplicons with a mix of HASPB1 and HASPB2 motifs within these PCR products, indicating a structural reorganisation of HASPB in these strains. Similarly, Haralambous et al [30] in K26, a homologue of HASPB, also found unexpected amplicons for the Hussen strain as compared to Gebre1 and Gilani. Nevertheless, despite this structural reorganisation, there was limited HASPB amino acid diversity across the strains. There was only one polymorphism compared to rK28 that involved a charge change, Lys→Glu^{49} in HASPB2, potentially affecting antigenicity. A recent study [31] reports HASPB repeat sequence diversity in Indian *L. donovani* strains; a comparison with sequences derived here shows that among those East African strains producing unexpected sized amplicons (Table 4), there is the presence of repeat sequence types that are also predominant among Indian strains (PKEDDHAHNDGGG, PKEDGHTQKNDGDG, PKEDDHAHNDGDG). In addition we find repeat types not reported from Indian strains, for example repeats beginning with Lys in place of Pro (Hussen, UGX-Marrow, SUKKAR 2), or ending in Val (LRC-L57, MRC(L)3).

We did not have patient samples from which to attempt direct amplification of kinesin and HASPB and confirm diversity *in situ*. Nevertheless the polymorphisms described here do not occur stochastically across strains but at sites of reported kinesin diversity, or in the case of the HASPB2, are consistent among strains (Ala^{32}-Gly-Val^{33} or Pro→Gln^{96}). Furthermore, the strains were cryopreserved, not subjected to prolonged passage *in vitro*, and sequences were determined bi-directionally and/or repeated to confirm their validity.

We have undertaken the most comprehensive analysis of diagnostic kinesin and HASPB antigen diversity in East African strains to date. We show that there is extensive kinesin genetic diversity among strains and between East Africa and South Asia, with ample scope for influencing performance of rK39 diagnostic assays. Future research should both widen and focus the genomic comparisons between strains and also compare immune competence profiles among East African, Brazilian and South Asian populations as an alternative or contributory factor to variable RDT performance. There are broader implications from these findings. Firstly, we see the crucial importance of sustaining accessible collections of *Leishmania* strains representative of genetic lineages and global diversity. Secondly, due to present limitations in the capacity of whole genome sequencing to assemble complex antigen gene families, especially those comprised of repeated sequences, targeted analysis of individual strains is also necessary for these comparisons of genetic diversity. Thirdly, it is evident that comparative genomics has a vital role in guiding the optimisation of recombinant or synthetic diagnostic antigens. Not only are even better diagnostic tests needed for diagnosis of VL but biomarkers are urgently required to distinguish symptomatic cases, asymptomatic infections at risk or not of progression to VL, and post-treatment outcome (relapse *versus* cure).

Acknowledgments

We thank Isabel Mauricio and Rania Baleela for guidance on choice of strains, Matthew Yeo for contributing to parasite culture, and for their constructive comments on the manuscript. We also thank François Chappuis for his comments. The authors are also grateful to anonymous reviewers for their improvements to the manuscript.

Author Contributions

Conceived and designed the experiments: TB MAM. Performed the experiments: TB. Analyzed the data: TB MAM. Contributed reagents/materials/analysis tools: TB MAM. Wrote the paper: TB MAM MB. Coordinated funding application and the NIDIAG associated research partnership: MB.

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PUBLICATIONS ARISING: *Leishmania donovani* complex and visceral leishmaniasis

ANNEX 4: **Bhattacharyya T***, Bowes DE*, El-Safi S, Sundar S, Falconar AK, Singh OP, Kumar R, Ahmed O, Boelaert M, Miles MA (2014). Significantly lower anti-*Leishmania* IgG responses in Sudanese versus Indian visceral leishmaniasis. *PLoS Negl Trop Dis* 8 e2675

Key points, novel results and implications

- As described in ANNEX 4 Bhattacharyya *et al*., 2013 recent multi-centre trials of the rK39 RDT have shown much lower sensitivities in East Africa than the South Asia, and the extent and nature of *Leishmania* antigen diversity was examined as a possible cause.
- Here, the relevance of the human immunoglobulin response by patients from endemic regions in Sudan and India is examined by comparative serology against *Leishmania* lysates originating from both regions.
- ELISA plates were coated with whole cell lysates of Sudanese and Indian *L. donovani* strains, such that each strain was coated on half of each plate. VL patient plasma from the different endemic areas of Bihar (India) and Gedaref (Sudan) were then titrated aginst the lystaes, for comparasion of anti-*Leishmania* response.
- Composite results for each cohort (India n=36, Sudan n=36) were analysed by plotting 1/log plasma dilution of the serial titration against ELISA readout. A striking finding was that the Sudanese cohort produced much lower IgG titres than the Indian, as measured by 50% end titres. This was observed regardless of antigen source, patient age or sex ($p<0.0001$).
- This work shows that a possible reason for the lower sensitivity of the rK39 RDT in East Africa is the lower IgG response generated, which may be due to genetic/ethnic, nutritional or environmental factors.

Candidate's contribution:

The candidate prepared the antigen and co-performed, via supervision of a LSHTM Masters degree research student project, the ELISAs described in the publication. The candidate prepared the first draft of the manuscript, which was accepted for publication by *PLoS Negl Trop Dis* in December 2013 following academic peer review.

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Significantly Lower Anti-*Leishmania* IgG Responses in Sudanese versus Indian Visceral Leishmaniasis

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Abstract

Background: Visceral leishmaniasis (VL), a widely distributed systemic disease caused by infection with the *Leishmania donovani* complex (*L. donovani* and *L. infantum*), is almost always fatal if symptomatic and untreated. A rapid point-of-care diagnostic test for anti-*Leishmania* antibodies, the rK39-immunochromatographic test (rK39-ICT), has high sensitivity and specificity in South Asia but is less sensitive in East Africa. One of the underlying reasons may be continent-specific molecular diversity in the rK39 antigen within the *L. donovani* complex. However, a second reason may be differences in specific IgG anti-*Leishmania* levels in patients from different geographical regions, either due to variable antigenicity or immunological response.

Methodology/Principal Findings: We determined IgG titres of Indian and Sudanese VL patients against whole cell lysates of Indian and Sudanese *L. donovani* strains. Indian VL patients had significantly higher IgG titres against both *L. donovani* strains compared to Sudanese VL patients ($p<0.0001$). Mean reciprocal $\log_{10}$ 50% end-point titres ($1/\log_{10}$d) were i) 3.80 and 3.88 for Indian plasma and ii) 2.13 and 2.09 for Sudanese plasma against Indian and Sudanese antigen respectively ($p<0.0001$). Overall, the Indian VL patients therefore showed a 46.8–61.7 -fold higher mean ELISA titre than the Sudanese VL patients. The higher IgG titres occurred in children (<16 years old) and adults of either sex from India (mean $1/\log_{10}$d: 3.60–4.15) versus Sudan (mean $1/\log_{10}$d: 1.88–2.54). The greatest difference in IgG responses was between male Indian and Sudanese VL patients of ≥ 16 years old (mean $1/\log_{10}$d: 4.15 versus 1.99 = 144-fold ($p<0.0001$).

Conclusions/Significance: Anti-*Leishmania* IgG responses among VL patients in Sudan were significantly lower than in India; this may be due to chronic malnutrition with Zn^{2+} deficiency, or variable antigenicity and capacity to generate IgG responses to *Leishmania* antigens. Such differential anti-*Leishmania* IgG levels may contribute to lower sensitivity of the rK39-ICT in East Africa.

Citation: Bhattacharyya T, Bowes DE, El-Safi S, Sundar S, Falconar AK, et al. (2014) Significantly Lower Anti-*Leishmania* IgG Responses in Sudanese versus Indian Visceral Leishmaniasis. PLoS Negl Trop Dis 8(2): e2675. doi:10.1371/journal.pntd.0002675

Editor: Peter C. Melby, University of Texas Medical Branch, United States of America

Received August 28, 2013; Accepted December 17, 2013; Published February 20, 2014

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Funding: This work is part of the NIDIAG network research partnership supported by the European Commission under the Health Cooperation Work Programme of the 7th Framework Programme (Grant agreement no. 260240, http://cordis.europa.eu/fp7/home_en.html). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The great majority of the estimated 200,000 to 400,000 annual new cases of visceral leishmaniasis (VL) occurs in six countries, with India having the highest estimated incidence in the world (146,700 to 282,000/year), Sudan having the highest in Africa (15,700 to 30,300/year) and Brazil having the highest in the Americas (4,200 to 6,300/year) [1]. In South Asia and East Africa, VL is caused by the kinetoplastid protozoan *Leishmania donovani*, transmitted by the sandfly vectors *Phlebotomus argentipes* in South Asia and *P. orientalis* and *P. martini* in East Africa. Following inoculation into the human host, the parasite disseminates through the lymphatic and vascular systems. Some infected individuals remain asymptomatic, but full-blown symptomatic VL with bone marrow infiltration and hepatosplenomegaly is almost always fatal if untreated [2].

The demonstration of *L. donovani* amastigotes in lymph node, spleen or bone marrow tissue smears is the definitive diagnostic method for infection, however due to the invasive nature and the operational difficulties associated with these procedures, serological assays have been developed. Serological (anti-*Leishmania* antibody) tests include the enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT) and the direct agglutination test (DAT) [3,4]. However, these antibody detection tests remain positive for several months to years after drug treatment and cure and therefore cannot readily diagnose relapse;

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To further serological studies on VL, and in the context of the existing literature, the relationship between IgG subclass responses in VL patients of different clinical status was an additional area of interest. That work is described in the following section (ANNEX 5 Bhattacharyya *et al*., 2014).

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Editor: Peter C. Melby, University of Texas Medical Branch, United States of America

Received August 28, 2013; **Accepted** December 17, 2013; **Published** February 20, 2014

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Author Summary

Visceral leishmaniasis (VL) is a systemic disease with highest prevalence in South Asia, East Africa, and Brazil. VL is caused by protozoan parasites of the *Leishmania donovani* complex, transmitted to humans when an infected sandfly takes a bloodmeal. Within the human host, the parasites replicate within cells, particularly of bone marrow and spleen. Without effective treatment, symptomatic VL is usually fatal. Correct treatment depends on accurate diagnosis, which is by detection of parasites or specific antibodies. The rK39 rapid diagnostic test for antibody is highly sensitive in South Asia but less so in East Africa, for poorly understood reasons. Here, we have directly compared the anti-*Leishmania* antibody response in groups of VL patients from India and Sudan. We found a strikingly higher anti-*Leishmania* antibody response in Indian compared to Sudanese patients, which was also seen when further analysed by age and sex of the patients. Thus in addition to parasite factors, we have shown that difference in antibody levels may contribute to the lower sensitivity of antibody-based diagnosis for VL in Sudan.

such tests can also be positive in asymptomatic individuals living in endemic areas and exposed to *L. donovani* infection yet with no history of VL or subsequent progression to VL.

The lateral-flow rapid diagnostic 'point-of-care' immunochromatographic test (ICT) format based on the rK39 antigen derived from a Brazilian isolate of *Leishmania infantum* (historically known as *L. chagasi*) [5] has demonstrated high levels of sensitivity in South Asia but is less effective in East Africa for diagnosis of VL [6,7]. A new ICT, based on the synthetic gene rK28, has recently been developed to overcome these limitations [8,9], and is currently under evaluation. Underlying explanatory factors for the different levels of rK39 diagnostic success observed across geographical regions may be molecular divergence between East African *L. donovani* kinesin gene homologues and the Brazilian *L. infantum* (*L. chagasi*)-derived rK39 sequence [10], and/or may be due to quantitative differences in the IgG titres generated against the rK39 antigen between South Asian and East African VL-endemic populations. Here, we compare anti-*L. donovani* IgG titres in cases of active VL in children and adults of each sex from India and Sudan against whole cell lysates of *L. donovani* strains from both countries. We find striking differences between the anti-*Leishmania* IgG titres of the two human populations.

Methods

Ethics statement

In India, comparative serology was approved by the Ethics Committee of the Banaras Hindu University, Varanasi, India. In Sudan the protocols were approved by the Ethical Research Committee, Faculty of Medicine, University of Khartoum and the National Health Research Ethics Committee, Federal Ministry of Health. Written informed consent was obtained from all adult subjects included in the study, or from the parents or guardians of individuals less than 18 years of age. This research was also covered by the London School of Hygiene and Tropical Medicine Ethics Committee approval of the EC NIDIAG project.

Study populations

Sudan: plasma samples were obtained upon clinical presentation from active cases of VL in the Gedaref region in eastern Sudan between July and September 2011. Patients were diagnosed as positive for VL by a combination of bone marrow aspiration, lymph node aspiration, or serology. India: plasma samples were obtained upon clinical presentation from active cases of VL in Bihar state, north-eastern India after 2009. Active VL patients were diagnosed by identification of parasitologically-positive splenic aspirates. All samples in Sudan were transported by continuous cold chain with samples maintained at 4°C, to the research laboratory at Suba University Hospital and there stored at −80°C; similarly all samples in India were cold chain transported at 4°C to the laboratory at Banaras Hindu University and stored at −80°C.

Antigen preparation

Strains of *L. donovani* originating from Sudan (MHOM/SD/97/LEM3458) and India (MHOM/IN/80/DD8) were cultured in αMEM (Sigma, UK) supplemented as described [11]. Mid-to-late log phase cultures were washed three times in phosphate-buffered saline (PBS), followed by three cycles of flash-freezing in liquid nitrogen and thawing in a cold water bath. Subsequently, cells were subjected to three 30 sec 12-micron sonication cycles on ice at 30 second intervals using a Soniprep 150 sonicator (MSE, UK). Sonicates were centrifuged at 12000×*g* for 1 min, and the supernatant used as antigen. Protein concentrations in these lysates were determined using the BCA Protein Assay kit (Fisher Scientific, Loughborough, UK).

Anti-*Leishmania donovani* ELISA

The indirect *L. donovani* ELISA was performed using relatively low antigen concentrations (0.2 µg/well) established by prior ELISA checkerboard titrations (not shown), with the intention of increasing ability to discriminate between patients generating high and low IgG antibody responses. Lysates of the *L. donovani* DD8 (Indian) and LEM3458 (Sudanese) strains, diluted to 2 µg/ml in 35 mM $NaHCO_3$/15 mM $NaCO_3$ buffer (pH 9.6), were separately added at 100 µl/well to 48 wells (A to H 1–6 or A to H 7–12) (see Figure 1) of Immulon 4HB ELISA plates (VWR, Lutterworth, UK) and incubated overnight at 4°C. After washing the plates three times using PBS containing 0.05% (vol/vol) Tween 20 (Sigma, Gillingham, UK) (PBST), they were blocked using 200 µl/well PBST containing 2% skimmed milk powder (Premier International Foods, Spalding, UK) (PBSTM) at 37°C for 2 hr. After washing three times with PBST, serial four-fold 1:400 to 1:25,600 dilutions of VL plasma samples (Table 1, Figure 1) (100 µl/well) in PBSTM were added to the plates and incubated at 37°C for 1 hr. Plasma samples from the two endemic countries were assayed on the same plate against antigens of both Indian and Sudanese strains and were matched for sex and age groups as shown in Figure 1. Following six PBST washes, a 1:5,000 dilution of peroxidase-labelled goat anti-human IgG (H+L) (Jackson ImmunoResearch, West Grove, USA) prepared in PBSTM was added at 100 µl/well and the plates incubated at 37°C for 1 hr. Following six PBST washes, 50 mM phosphate/citrate buffer (pH 5.0) containing 2 mM *o*-phenylenediamine HCl and 0.007% (vol/vol) H_2O_2 (Sigma, UK) was added at 100 µl/well and incubated in the dark at room temperature for 15 minutes. The substrate reactions were then stopped by the addition of 2 M H_2SO_4 (50 µl/well) and the ELISA plates were read at 490 nm (Spectra Max 190, Molecular Devices, Sunnyvale, USA). Coefficients of variation (mean positive control readings (n = 4)/standard deviation of positive control x 100) at dilutions of 1/400 and 1/1600 were calculated from simultaneous duplicate plates for both Indian and Sudanese sera (most coefficients of variation were less than 1%). Reference positive plasma controls were also used on every plate (Figure 1). Samples that gave coefficients of

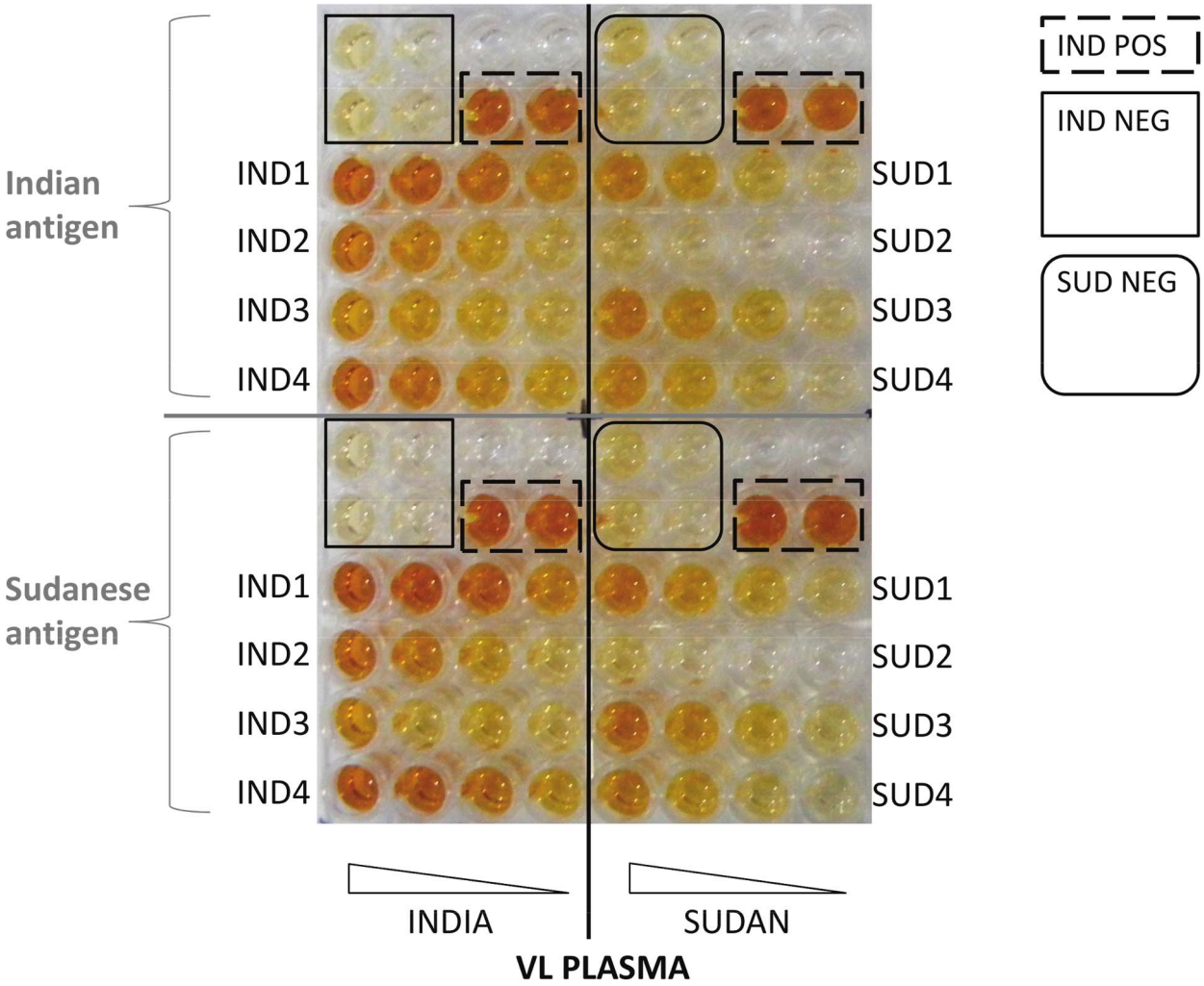

Figure 1. Design of assay for comparative ELISA serology. Serial four-fold dilutions of plasma samples from Indian and Sudanese VL patients were reacted with whole cell lysates of *L. donovani* strains isolated from each country. Identically formatted plates were run in all cases. Unlabelled wells were not used.
doi:10.1371/journal.pntd.0002675.g001

variation above 20% were repeated. Representative plasma samples from Indian and Sudanese endemic healthy controls and from cases of active VL were used as inter-assay controls. These plasma samples were chosen in preliminary assays as having median responses against the lysate obtained from the *L. donovani* strain isolated from the corresponding region (data not shown). To

Table 1. Indian and Sudanese plasma study populations used in comparative serology against *Leishmania donovani* antigens.

Endemic region	Sex	Age (years)	n	Mean age in years (range; standard deviation)
India (n = 36)	Male	≥16	10	33.4 (16–70; 17.45)
		<16	8	12.8 (10–15; 1.67)
	Female	≥16	8	30.5 (17–52; 12.22)
		<16	10	12.8 (9–15; 2.20)
Sudan (n = 36)	Male	≥16	10	22.2 (16–43; 9.17)
		<16	8	9.8 (4–15; 4.1)
	Female	≥16	7	35.6 (25–60; 12.35)
		<16	11	8.1 (1–15; 4.55)
Total n = 72				

doi:10.1371/journal.pntd.0002675.t001

maximize the chances of identifying differences in the IgG responses against the two different *L. donovani* strains (Figure 2) and between Indian and Sudanese VL patients of each sex and age group (≥ or <16 years old), the highest mean absorbance (Abs_{max}) value was determined for each data set, from which the $Abs_{max}/2$ was determined for calculating the mean reciprocal $\log_{10}$ 50% end-point ($1/\log_{10}t_{50}$) ELISA titres. This methodology has been recommended for comparisons between high, low and non-classical IgG responses of acute and convalescent phase samples from both individuals and patient groups infected with another pathogen [12,13]. This approach is more accurate than determining ELISA titres as either: i) single end-point values located on the very trailing slope of the ELISA sigmoid curve (e.g. values that are 2 standard deviations above the mean obtained using negative control sera) or ii) the mean absorbance values obtained using single serum dilutions (e.g. 1/100) [12]. In the latter case, the limited use of absorbance values results in a low dynamic range of data for comparisons [12].

Statistical analyses

Two sided independent sample t-tests were used to analyse the data (SPSS INC. Armonk, NY: IBM Corp). Normality was assessed using a three tiered approach. Shapiro-Wilk tests were conducted first with subsequent evaluation of the data through visual assessment and by calculating a z-score for skewness ($Z_{Skewness}$ = Skewness-0/SE $_{Skewness}$) as proposed by Ghasemi and Zahediasl [14]. In the event of a violation of the assumption of homogeneity of variance (homscedasticity), SPSS calculated a corrected p value. Significance was set at the 5% level.

Results

Table 1 summarises the age and sex compositions of the Indian and Sudanese VL patients who provided plasma for comparisons of serological responses between the two endemic regions.

Comparison of Indian and Sudanese IgG isotype responses

The mean overall Sudanese (n = 36) and Indian (n = 36) active VL patients' IgG responses, at each plasma dilution, against lysates of *L. donovani* strains isolated from each endemic area, with 95% confidence intervals (CIs) are shown in Figure 2. In a few instances, the unforeseen low Sudanese titres required minor extrapolation of curves to determine specific $1/\log_{10}50\%$ end-point titres but since homoscedasticity was obtained in each case a correction factor was not required. Similar high mean maximum absorbance (Abs_{max}) values were obtained for the Indian VL patients against both the Indian and Sudanese *L. donovani* strains. However the Indian VL patients showed significantly higher IgG titres against both the Sudanese (mean $1/\log_{10}t_{50}$: 3.88) and Indian (mean $1/\log_{10}t_{50}$: 3.80) *L. donovani* strains than the Sudanese VL patients (mean $1/\log_{10}t_{50}$: 2.09 and 2.13 respectively) (Figure 2A and B, Table 2; two-sided independent sample t-tests for both *L. donovani* strains: $p<0.0001$). Thus, overall the Indian VL patients generated 46.8–61.7-fold higher IgG titres than the Sudanese patients against the *L. donovani* strains (Table 2). We further assessed whether there were differences in anti-*L. donovani* IgG titres generated by the male and female Indian and Sudanese VL patients of less than or greater than 16 years old against both *L. donovani* strains. We again used the mean highest absorbance (Abs_{max}) value for each data set (Figure 3A to D) to maximize the identification of differences in the mean IgG responses, from which the $Abs_{max}/2$ value was determined for interpolating the mean $1/\log_{10}t_{50}$ IgG ELISA titres. We observed that the male VL patients of ≥16 years old from India showed higher Abs_{max} values against both the Sudanese (Abs_{max} 3.34) and Indian (Abs_{max} 3.33) antigens than the female Indian VL patients of the same age group (Abs_{max} 3.11 and 2.99, respectively) (Figure 3C and D). This difference was less in the male and female Indian VL patients of <16 years old (Figure 3A and B). Importantly, the Sudanese male and female children (<16 years

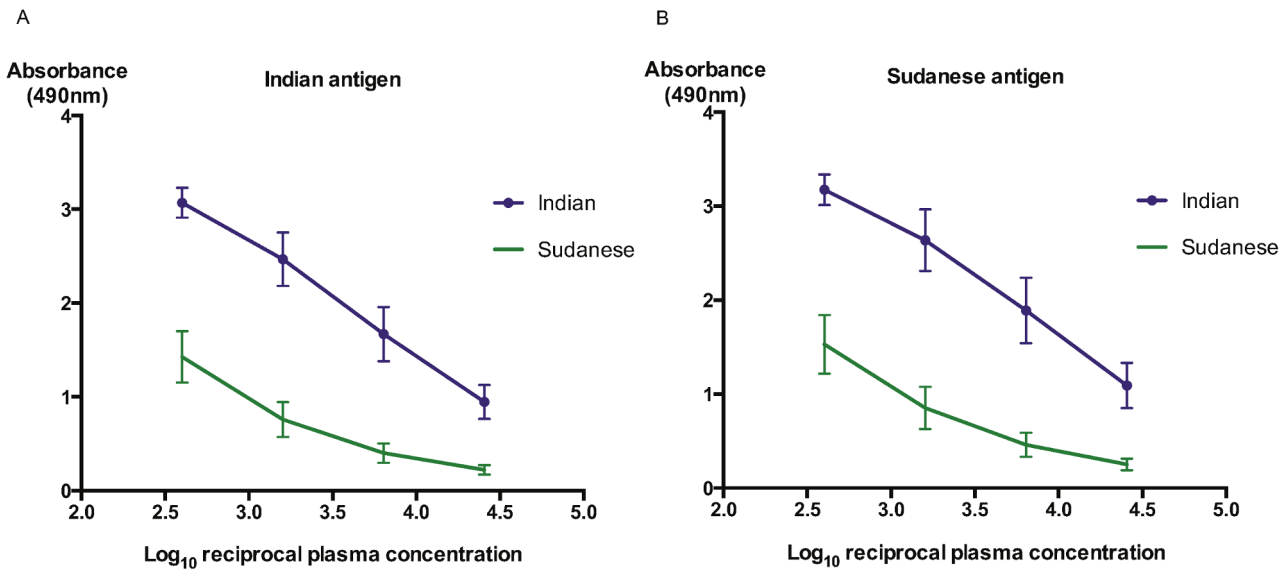

Figure 2. IgG anti-*Leishmania* responses are higher in Indian than Sudanese VL patients. The mean IgG responses are shown with 95% CI, for 36 Indian (purple line with nodes) and 36 Sudanese (green line) patients with active VL, against lysates of *L. donovani* strains isolated from [A] India or [B] Sudan. Comparative mean $1/\log_{10}t_{50}$ IgG titres and fold-differences are shown in Table 2. Statistical p values of <0.0001 were obtained for both the Indian and Sudanese antigens.
doi:10.1371/journal.pntd.0002675.g002

Table 2. Mean reciprocal ELISA titres and fold differences of Indian and Sudanese VL patients by age, sex and antigen source.

				Mean reciprocal 50% end-point titre ($1/\log_{10}t_{50}$) interpolated from $Abs_{max}/2$				
Figure	Sex	Age	Antigen source	Indian plasma	Sudanese plasma	Fold difference	p value (95% CI)	p value (95% CI)
						(Indian-Sudanese)	Sex: M or F	Both Sexes
2A	Both	All	Sudan	3.88	2.09	61.7	-	p<0.0001 (1.35–2.24)
2B	Both	All	India	3.80	2.13	46.8	-	p<0.0001 (1.29–2.06)
3A	M	<16	Sudan	3.63	2.52	12.9	p<0.007 (0.356–1.87)	p<0.0001 (0.929–2.21)
	F	<16	Sudan	3.78	1.88	79.4	p<0.001 (0.898–2.91)	
3B	M	<16	India	3.60	2.54	11.5	p<0.004 (0.385–1.73)	p<0.0001 (0.90–2.03)
	F	<16	India	3.69	1.92	58.9	p<0.001 (0.883–2.66)	
3C	M	≥16	Sudan	4.15	1.99	144	p<0.0001 (1.45–2.87)	p<0.0001 (1.38–2.67)
	F	≥16	Sudan	3.95	2.09	72.4	p<0.02 (0.388–3.33)	
3D	M	≥16	India	4.03	2.02	102	p<0.0001 (1.53–2.48)	p<0.0001 (1.36–2.40)
	F	≥16	India	3.84	2.13	51.3	p<0.008 (0.539–2.89)	

doi:10.1371/journal.pntd.0002675.t002

old) and adults all generated lower mean IgG ELISA titres against both *L. donovani* strains than the corresponding Indian VL patient sex/age groups (Figure 3A to D). These differences ranged from 11.5 to 144-fold (Table 2).

The Sudanese male VL patients less than 16 years old showed the lowest fold-differences compared to the Indian male VL patients of the same age group (12.9-fold and 11.5-fold against Sudanese and Indian antigen respectively). In contrast, the Sudanese male VL patients older than 16 years showed the highest fold-difference with the Indian male patients of the same age group (144-fold).

Discussion

According to a recent report from the World Health Organisation, among the research priorities for human diseases caused by infection with kinetoplastid protozoa is research on diagnostics for case detection and characterisation [15]. For many years, the rK39 antigen has been the only rapid diagnostic ICT in a lateral flow system that is readily applicable for field diagnosis of VL and that can be used with minimal training with no other equipment or reagent. Despite high levels of sensitivity in South Asia, the rK39 ICT has shown lower efficacy in East Africa, for reasons that are not fully understood. The recent rK28 ICT, based on 2×39 amino acid repeats of a Sudanese *L. donovani*-derived kinesin homologue of rK39, flanked by HASPB sequences, has been developed in an attempt to overcome the differential sensitivity of rK39. Reduced efficacy in East Africa has also been reported for a diagnostic test using another *L. donovani* antigen, rKE16 [7].

Different sensitivities of the rK39 ICT in South Asia and East Africa may be explained by molecular divergence in diagnostic antigen sequences of geographically disparate *L. donovani* strains and/or reflect the different levels of overall IgG anti-*Leishmania* response between human populations in VL endemic areas. We have assessed the first of these factors, and demonstrated substantial and regional specific antigen diversity [10], an observation subsequently reported by others [16].

Differential serological responses among different ethnic groups within the same geographical region have been reported previously, for both bacterial and protozoal pathogens, although generally without understanding of the genetic/biological explanations. Higher responses were found against: *Helicobacter pylori* in non-Japanese Brazilians than Japanese Brazilians [17]; *Streptococcus pyogenes* superantigen in Polynesian than New Zealand Europeans [18]; *Plasmodium falciparum* in Fulani than sympatric ethnic groups in Burkina Faso [19]; *P. falciparum* in Austro-Asiatic than Tibeto-Burman groups in north-east India [20]. Jensen et al [21] reported comparatively higher anti-*Plasmodium* titres in subjects from Flores (Indonesia) than in counterparts from southern Sudan. In the UK, higher IgG levels were found in South Asian patients compared to European counterparts in the context of cardiovascular disease [22].

Here, we used comparative analysis of serological responses, as applied elsewhere [12,13] to assess accurately differences in IgG titres between VL patient cohorts from Bihar (India) and Gedaref (Sudan). We have clearly shown that active VL patients from India generated significantly higher anti-*L. donovani* IgG responses against whole cell lysates of both Indian and Sudanese parasite strains than active VL patients from Sudan. This may contribute to the reduced sensitivity of the commercial rK39 ICT assay with Sudanese versus Indian VL patients. We used soluble antigens from *Leishmania* whole cell lysates in the comparative ELISAs. On western blots human VL serum antibodies recognise multiple antigens in such lysate preparations (data not shown). It is therefore likely that Sudanese patients with active VL had reduced IgG titres against several *L. donovani* promastigote antigens; we have not yet determined whether the response to particular antigens is depleted. Thus, it may be challenging to identify *L. donovani* antigens that provide adequately high sensitivity and specificity for East Africa. Whilst it would be of interest to extend these comparisons to include IFAT, results from ELISA and IFAT generally accord, and we therefore anticipate that the two tests will give compatible data [23]. Detection of antigens in urine may provide an alternative, non invasive approach, possibly giving prognostic information [24,25]. In the same multi-centre trial comparing rK39 dipstick sensitivity and specificity across South Asia and East Africa, Boelaert et al. [6] also compared the KAtex® test, which detects a *Leishmania* carbohydrate antigen in urine

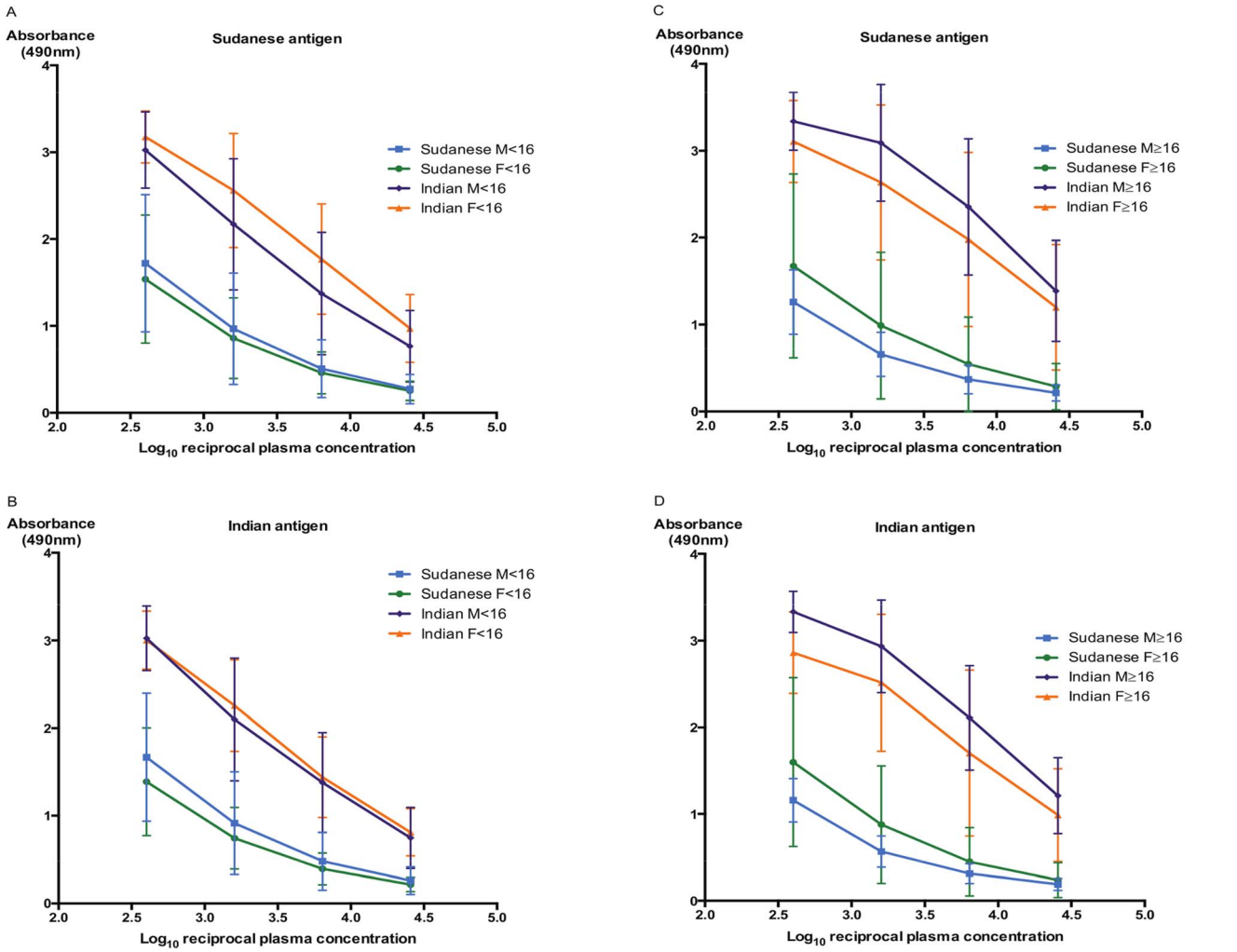

Figure 3. IgG anti-*Leishmania* responses are higher in Indian VL regardless of age, sex or antigen source. The mean IgG responses and 95% CI are shown for Indian (purple and orange lines) and Sudanese (blue and green lines) active VL patients against lysates of *L. donovani* strains isolated from Sudan [A & C] or India [B & D]. Comparative mean $1/\log_{10}t_{50}$ IgG titres and fold-differences are given in Table 2.
doi:10.1371/journal.pntd.0002675.g003

[26,27]. Sensitivities in both regions were described as moderate to very low, with the lowest in India and Nepal, although higher sensitivities have been recently reported from Bangladeshi studies [28–30].

We have not yet assessed antibody levels in patients with post-kala azar dermal leishmaniasis (PKDL). This is of interest because PKDL is a sequela of VL that displays markedly different epidemiological features between regions, in Sudan occurring at a higher frequency (>50%) and much sooner after VL than in India (<10%) [31]. In Sudan and India the plasma was collected at time of clinical presentation of VL and no obvious differences in symptoms between the two cohorts were observed; further comparisons would benefit from more precise grading of clinical severity.

Whilst the active VL patient numbers used in our analysis were relatively low, Indian males ≥16 years old with active VL generated higher IgG responses than females of the same age group, whereas Sudanese male VL patients ≥16 years old generated lower IgG responses than females of the same age group. These trends were not apparent in Indian or Sudanese patients <16 years old. Differences in infection between males and females in late teenage years and in adults may be attributable to increased susceptibility of young and adult men [32]; testosterone increases *L. donovani* infection in macrophages [33]. In Sudan VL is a disease of extreme poverty with high incidence associated with chronic malnutrition, displacement of populations and lack of health care [1,34–39]. Particular ethnic groups, possibly with greater genetic susceptibility, may be more affected within the ethnically mixed Sudanese population [40,41].

Malnutrition is known to reduce both human innate and acquired immune responses against parasitic diseases such as VL [42,43]. Experimental models of protein, zinc and iron malnutrition have shown reduced immune responses in *L. infantum* infected mice and increased early visceralisation [44,45]. Of these, even mild zinc deficiency has been extensively reported to: i) reduce T- and B -cells in the blood and lymphoid tissues of humans, non-human primates and other animals, ii) dramatically reduce both T- and B- cell numbers in the bone marrow by >75%, iii) dramatically reduced B-cell IgG production, particularly to T-dependent antigens, by 90% and iv) reduce both T- and B- cell proliferation, resulting in depletion through apoptosis [46]. More specifically, zinc deficiency in mice was shown to block the

development of bone-marrow pre-B and immature B-cells, resulting in reduced B lymphocytes in the spleen; pre-natal zinc deficiency in monkeys and mice reduced lymphocyte numbers and IgG concentrations and produced long-term suppression of IgM, IgA and IgG. Zinc deficiency in mice also resulted in reduced B-cell responses to recall antigens with which they had previously been inoculated, thereby suggesting that T- and B- cell depletion resulted in impaired immunological memory [46]. Thus, malnutrition with zinc deficiency, even if relatively mild and previously encountered may play an important role in the significantly lower IgG titres of the Sudanese patients.

Human interleukin-2 (IL-2) and signalling through its receptor (IL-2R) play a critical role in both T- and B-cell proliferation and induce the expression of the *IL-2Rα* gene in B cells, thereby increasing B-cell responses to IL-2, antibody heavy and light chain expression and antibody secretion [47]. Thus, single nucleotide polymorphisms in the genes encoding IL-2 and its receptor (IL-2Rα or IL-2Rβ) chains significantly affect human IgG titres in response to both inactivated and live attenuated vaccines (e.g. the live attenuated measles-mumps-rubella (MMR) vaccine) [48,49]. While there have not been similar studies performed on Sudanese populations, VL patients in eastern Sudan had a significant association with two SNPs in the *IL-2Rβ* gene, one of which encoded a non-conserved amino acid substitution (G245R) in the proximal membrane domain that was very likely to affect IL-2 signalling [50]. Thus, these SNPs and others located in the genes that affect IL-2 expression, function and responses may have contributed to the reduced IgG responses of Sudanese VL patients observed in our study.

Human leukocyte antigen (HLA) polymorphisms in the genes encoding the MHC class II (HLA-DR, -DP and -DQ) molecules are known to have significant effects on IgG responses to vaccines [48], infectious diseases, cancer, asthma and autoimmune diseases [51–53]. Early studies did not however find a significant association of HLA class II polymorphisms in VL patients in either Tunisia or Brazil [54,55]. By contrast, in a more recent study performed using VL patients' samples from Brazil and India, *HLA-DRB1* and *HLA-DQA1* were the only genes associated with VL risk in both populations [56] but comparisons of anti-*L. donovani* IgG titres between these groups or the control groups were not performed. Since no similar HLA studies have been performed on Sudanese VL patients, it is presently not yet known whether polymorphisms in these genes may have contributed to the significantly lower IgG titres observed in the Sudanese versus Indian VL patients found in our study.

Thus, the significant differences in the anti-*L. donovani* IgG titres found here between the Indian and Sudanese VL patients in this study may result from a combination of environmental and genetic factors, in which malnutrition with Zn^{2+} deficiency, differences in IL-2 responses due to polymorphisms in *IL-2* and its receptor (*IL-2Rα* and *IL-2Rβ*) genes and possibly *HLA-DRB1* and *HLA-DQA1* genes are to date the most likely candidates.

We have presented here a direct ELISA comparison of serological responses between VL patients from different endemic regions (Sudan and India), and have shown a clear difference in the levels of IgG anti-*Leishmania* antibodies. Comparative serological responses could also be extended to samples from Brazilian cases of VL to investigate their contribution to reported lower sensitivity with rK39-based ICTs [7], despite this diagnostic antigen being derived from a Brazilian *L. infantum* (*L. chagasi*), dispersed from Iberian Europe [57]. Our data suggest that the relative levels of ability to mount a humoral response against *Leishmania* antigen between the different human populations may be a significant contributory factor in the differing levels of sensitivity reported for rapid diagnostic tests applied in both regions, and argue for the design and development of a test more suited to East Africa, guided by comparative genomics [10].

Acknowledgments

We thank Juerg Utzinger for critical reading and improvement of the manuscript. The authors are also grateful to anonymous reviewers for their improvements to the manuscript.

Author Contributions

Conceived and designed the experiments: TB AKF MAM. Performed the experiments: TB DEB OPS RK. Analyzed the data: TB DEB AKF MAM. Contributed reagents/materials/analysis tools: SES SS OPS RK OA. Wrote the paper: TB DEB AKF MAM MB. Coordinated funding application and the NIDIAG associated research partnership: MB.

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PUBLICATIONS ARISING: *Leishmania donovani* complex and visceral leishmaniasis

ANNEX 5: **Bhattacharyya T**, Ayandeh A, Falconar AK, Sundar S , El-Safi S, Gripenberg MA, Bowes DE, Thunissen C, Singh OP , Kumar R, Ahmed O, Eisa O, Saad A, Pereira SS, Boelaert M, Mertens P, Miles MA. IgG1 as a potential biomarker of post-chemotherapeutic relapse in visceral leishmaniasis, and adaptation to a rapid diagnostic test. *PLoS Negl Trop Dis* 8 e3273

Key points, novel results and implications

- Current definitions of successful cure following chemotherapy for visceral leishmaniasis are based on clinical definitions. The identification of biomarkers which can indicate therapeutic outcome (cure or relapse), especially in the form of a point-of-care rapid assay, has been listed by WHO as a knowledge gap and research priority (WHO, 2012)
- Here, the association of IgG subclass with clinical status in VL was investigated by ELISA using Indian and Sudanese samples, initally from unpaired samples from pre-treatment patients, those deemed cured after therapy, and those deemed relapsed. Levels of IgG1 (and to a lesser degree IgG3) were elevated in the pre-treatment and relapsed groups, compared to cured ($p<0.0001$).
- When using paired pre- and post-treatment samples from patients considered cured, the decrease in IgG1 was not significant in the immediate few weeks following treatment, but was greatly diminished in the 6-month post treatment samples in cohorts compared to day 0 ($p = 0.0032$) or day 15 ($p<0.0001$).
- Two prototype lateral flow immunochromatographic RDTs were developed to detect IgG1 levels following VL treatment: more than 80% of the relapsed VL patients were IgG1 positive; at least 80% of the cured VL patients were IgG1 negative ($p <0.0001$).
- Thus this work demonstrated that six months after treatment of active VL, elevated levels of specific IgG1 were associated with treatment failure and relapse, whereas no IgG1 or low levels were detected in cured VL patients. A lateral flow RDT was successfully developed to detect anti-VL IgG1 levels as a potential biomarker of post-chemotherapeutic relapse.

Candidate's contribution:

The candidate prepared the antigen and co-performed, in supervision of LSHTM Masters degree research student projects, the ELISAs described in the manuscript. The candidate prepared the first draft of the manuscript, requiring the coherent compilation of a number of data sets and a comprehensive review of the literature, and which was accepted for publication by *PLoS Negl Trop Dis* in September 2014**.**

IgG1 as a Potential Biomarker of Post-chemotherapeutic Relapse in Visceral Leishmaniasis, and Adaptation to a Rapid Diagnostic Test

Tapan Bhattacharyya[1*], Armon Ayandeh[1], Andrew K. Falconar[2], Shyam Sundar[3], Sayda El-Safi[4], Marissa A. Gripenberg[1], Duncan E. Bowes[1], Caroline Thunissen[5], Om Prakash Singh[3], Rajiv Kumar[3,6], Osman Ahmed[4,7], Osama Eisa[4], Alfarazdeg Saad[4], Sara Silva Pereira[1], Marleen Boelaert[8], Pascal Mertens[5], Michael A. Miles[1]

Abstract

Introduction

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IgG1 as a Potential Biomarker of Post-chemotherapeutic Relapse in Visceral Leishmaniasis, and Adaptation to a Rapid Diagnostic Test

Tapan Bhattacharyya[1]*, Armon Ayandeh[1], Andrew K. Falconar[2], Shyam Sundar[3], Sayda El-Safi[4], Marissa A. Gripenberg[1], Duncan E. Bowes[1], Caroline Thunissen[5], Om Prakash Singh[3], Rajiv Kumar[3,6], Osman Ahmed[4,7], Osama Eisa[4], Alfarazdeg Saad[4], Sara Silva Pereira[1], Marleen Boelaert[8], Pascal Mertens[5], Michael A. Miles[1]

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Abstract

Background: Visceral leishmaniasis (VL), caused by protozoa of the *Leishmania donovani* complex, is a widespread parasitic disease of great public health importance; without effective chemotherapy symptomatic VL is usually fatal. Distinction of asymptomatic carriage from progressive disease and the prediction of relapse following treatment are hampered by the lack of prognostic biomarkers for use at point of care.

Methodology/Principal Findings: All IgG subclass and IgG isotype antibody levels were determined using unpaired serum samples from Indian and Sudanese patients with differing clinical status of VL, which included pre-treatment active VL, post-treatment cured, post-treatment relapsed, and post kala-azar dermal leishmaniasis (PKDL), as well as seropositive (DAT and/or rK39) endemic healthy controls (EHCs) and seronegative EHCs. *L. donovani* antigen-specific IgG1 levels were significantly elevated in relapsed *versus* cured VL patients ($p<0.0001$). Using paired Indian VL sera, consistent with the known IgG1 half-life, IgG1 levels had not decreased significantly at day 30 after the start of treatment ($p=0.8304$), but were dramatically decreased by 6 months compared to day 0 ($p=0.0032$) or day 15 ($p<0.0001$) after start of treatment. Similarly, Sudanese sera taken soon after treatment did not show a significant change in the IgG1 levels ($p=0.3939$). Two prototype lateral flow immunochromatographic rapid diagnostic tests (RDTs) were developed to detect IgG1 levels following VL treatment: more than 80% of the relapsed VL patients were IgG1 positive; at least 80% of the cured VL patients were IgG1 negative ($p<0.0001$).

Conclusions/Significance: Six months after treatment of active VL, elevated levels of specific IgG1 were associated with treatment failure and relapse, whereas no IgG1 or low levels were detected in cured VL patients. A lateral flow RDT was successfully developed to detect anti-*Leishmania* IgG1 as a potential biomarker of post-chemotherapeutic relapse.

Citation: Bhattacharyya T, Ayandeh A, Falconar AK, Sundar S, El-Safi S, et al. (2014) IgG1 as a Potential Biomarker of Post-chemotherapeutic Relapse in Visceral Leishmaniasis, and Adaptation to a Rapid Diagnostic Test. PLoS Negl Trop Dis 8(10): e3273. doi:10.1371/journal.pntd.0003273

Editor: Albert Picado, Barcelona Centre for International Health Research (CRESIB), Spain

Received June 29, 2014; **Accepted** September 14, 2014; **Published** October 23, 2014

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: This work is part of the NIDIAG network research partnership supported by the European Commission under the Health Cooperation Work Programme of the 7th Framework Programme (Grant agreement no. 260260, http://cordis.europa.eu/fp7/home_en.html). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: I have read the journal's policy and the authors of this manuscript have the following competing interests: The design of the lateral flow rapid diagnostic tests was performed as a research collaboration with Pascal Mertens and Caroline Thunissen of the company Coris BioConcept, Belgium, without payment; none of the authors have any financial, non-financial, professional or personal conflicting interests. This collaboration does not alter our adherence to all PLOS NTDs policies on shared data and materials.

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Introduction

The leishmaniases are widespread neglected infectious diseases of major public health importance, caused by protozoan parasites of the *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*) subgenera. There are two principal symptomatic clinical presentations of leishmaniasis: a) visceral (kala-azar, VL) caused by the *Leishmania donovani* complex, with circa 400,000 cases per year [1], which without appropriate chemotherapy is usually fatal, and b) cutaneous (CL) caused by diverse *Leishmania* species, some of which may give rise to diffuse cutaneous leishmaniasis (DCL) or metastatic mucocutaneous disease (MCL), the latter with devastating destruction of the nasopharynx [2]. The effective clinical management, chemotherapy and control of transmission of VL are

Author Summary

Visceral leishmaniasis (VL) is a systemic disease with highest prevalence in South Asia, East Africa, and Brazil. VL is caused by protozoan (unicellular) parasites of the *Leishmania donovani* complex, transmitted to humans when an infected sandfly takes a bloodmeal. Within the human host, the parasites replicate within cells, particularly of bone marrow and spleen. Without effective treatment, symptomatic VL is usually fatal. As outlined in a recent World Health Organisation report, the development of new diagnostic tools to test for successful cure after chemotherapy is a research priority. In this work we investigated the association of clinical status of VL patients (active pre-treatment, and those deemed cured or relapsed post-treatment) with subclasses of the IgG antibody response made to *L. donovani* infection. We show that high levels of subclass IgG1 are found in pre-treatment and relapsed patients, but are very much lower in patients deemed to be cured. We further show that the decrease in IgG1 is detectable in patients 6 months after successful treatment, and that this detection method can be adapted to a rapid diagnostic test format requiring minimal technical expertise. Thus we believe that IgG1 levels are potentially a biomarker of post-chemotherapeutic monitoring.

largely dependent on early and unequivocal diagnosis. Given that many VL patients live below the poverty threshold in remote areas poorly serviced by the health system, the diagnostic tools should be ASSURED (Affordable, Sensitive, Specific, User Friendly, Rapid, Equipment free and Deliverable where needed) [3]. The most sensitive and specific method to detect the causative agent of VL is microscopic examination of (invasive) spleen aspirates; bone marrow and lymph node aspirates provide similar high specificity but lesser sensitivity. More user-friendly point-of-care (POC) diagnostics have been developed based on antibody detection against rK39 and these provide high diagnostic accuracy in suspected first-time episodes of VL when combined with a clinical case definition [4–6]. However, these rapid diagnostic tests based on antibody detection are unable by themselves to distinguish asymptomatic carriers from those who will progress to acute fatal disease, and following chemotherapy they remain positive for many months precluding the detection of any relapse. To resolve the limitations of current diagnostic tools higher resolution, non-invasive, rapid and affordable POC tests are thus needed that define clinical status and indicate prognosis.

Current serological tests for VL include the enzyme linked immunosorbent assay (ELISA) with either crude or purified *Leishmania* promastigote antigens, the direct agglutination test (DAT) or indirect immunofluorescence test (IFAT) [7,8]. Each of these tests has disadvantages: the ELISA requires laboratory facilities and technical training; the DAT test has limited commercial access and involves several hours or overnight incubation before reading the results, and the IFAT uses a costly fluorescence microscope.

The recombinant repetitive antigen rK39, a product of the *Leishmania* kinesin-like gene, which was first cloned from Brazilian *Leishmania infantum* (synonym *L. chagasi*) [9] has been adapted to an immunochromatographic, lateral flow format and applied extensively as a rapid diagnostic test (RDT) for VL. However, in multicentre trials rK39 had much higher sensitivity in South Asia than in East Africa [10,11]. This could be due to the geographical diversity in the 6.5 repeats within the rK39 antigen sequence [12] and/or regional differences in anti-*Leishmania* IgG titres between the different human populations [13]. A modified recombinant derivative of rK39, designated rK28, which incorporated the first two rK39 repeats of a Sudanese kinesin flanked by HASPB1 and HAPB2 repeats, improved serological sensitivity for East African VL patients [14]. The ELISA, DAT, IFAT, rK39 and rK28 serological tests all rely on the detection of anti-*L. donovani* complex IgG. In human VL the IgG is produced secondarily, after IgM. IgG is divided into IgG1, IgG2, IgG3, and IgG4 subclasses, according to their ranked relative abundance in normal serum [15]. In general, human IgG1 and IgG3 are generated in response to protein antigens, whilst IgG2 and IgG4 are instead predominantly produced in response to polysaccharide antigens [15]. Previous studies on the humoral responses during active VL and after treatment, and on post kala-azar dermal leishmaniasis (PKDL), have been performed using samples from multiple endemic regions and a variety of serological tests [16–31]. Several of these studies, almost all of which used ELISAs, assessed anti-*L. donovani* complex titres of IgG, its subclasses and other Ig isotypes. In terms of IgG subclasses, increased IgG1 titres were identified in active disease (VL or PKDL) compared to healthy controls, and reduced IgG1 titres were reported after successful VL treatment. This published work suggested that the dynamics between proportionate IgG subclass responses and the clinical status of patients warranted further investigation to search for prognostic VL biomarkers.

A recent WHO report [8] designated the development of new diagnostics to determine cure as a research priority for VL, and there is an additional need for biomarkers to distinguish asymptomatic non-progressors from early progressive VL. Here we explore the potential of differential IgG subclass profiles to provide a biomarker for patients who relapsed after chemotherapy and therefore require urgent follow-up and life-saving additional or alternative chemotherapy. We show that the dynamics and levels of specific IgG1 responses can be indicative of relapse, and can be assessed using a simple, lateral flow immunochromatographic RDT format.

Methods

Ethics statement

In India, research on comparative serology and the collection of all serum or plasma samples was approved by the Ethics Committee of the Banaras Hindu University, Varanasi. Similarly, in Sudan research and collection of serum samples was approved by the Ethical Research Committee, Faculty of Medicine, University of Khartoum and the National Health Research Ethics Committee, Federal Ministry of Health. Written informed consent was obtained from all adult subjects included in the study or from the parents or guardians of individuals less than 18 years of age. This research was also approved, as part of the NIDIAG project, by the London School of Hygiene and Tropical Medicine Ethics Committee.

Sources of sera/plasma

Indian plasma samples were collected after 2007 from active VL, cured, relapsed, PKDL and asymptomatic groups from the endemic region of Muzaffarpur, Bihar state, north-eastern India, and control subjects from a region where VL is not endemic. In India active cases of VL were diagnosed parasitologically by microscopy of splenic aspirates. Sudanese serum samples were collected in 2011 and 2013, from active VL, treated, relapsed, PKDL, and endemic controls in the Gedaref region in eastern Sudan. All patients were HIV negative. Active cases of VL were diagnosed by a combination of microscopy of bone marrow or

lymph node aspirates in conjunction with serological assays. We have previously observed that serum and plasma derived from the same sample show no difference in titre in ELISA against *L. donovani* lysate (unpublished observations).

Unpaired samples. Table 1 shows the numbers of samples used in unpaired comparisons of IgG subclass responses and clinical status. For the Indian samples, two sets of comparisons were performed using these unpaired samples. There was an initial pilot study (Trial 1), followed by an expanded investigation (Trial 2), using different samples in each case. For the Sudanese sera, only one study was performed, after the Indian pilot serology.

Paired samples and multiple sequential samples. The comparative dynamics of IgG subclass response were studied using paired serum samples or, in the case of PKDL patients, several sequential samples from individual patients. Sera had been collected prospectively and the intervals between collections are shown in Table 2. For the Indian patients samples were available prior to treatment of active VL (day 0), paired with days 15, 30, or approximately 180 after start of treatment, depending on the patient. For Indian PKDL multiple sequential serum samples from the same patient were available at days 0, 30, 60, and 180 or 360 (Table 2). Paired sera from Sudanese patients were taken prior to treatment (day 0) and at the end of treatment lasting 11 days (ambisome), 17 days (sodium stibogluconate (SSG)+paromomycin) or 30 days (SSG alone).

Antigen preparation

L. donovani strain MHOM/IN/80/DD8 isolated from India, and MHOM/SD/97/LEM3458 isolated from Sudan, were cultured in αMEM (Sigma, UK) supplemented as described [32]. Mid-to-late log phase promastigote cultures were washed three times in phosphate-buffered saline (PBS), followed by three cycles of flash-freezing in liquid nitrogen and thawing in a 26°C water bath. Subsequently, these cells were subjected to three 30 seconds, 12-micron, sonication cycles on ice at 30 second intervals using a Soniprep 150 sonicator (MSE, UK). Sonicates were centrifuged at 12000× *g* for 1 minute at 4°C, and the supernatants were used as antigen. Protein concentrations in these lysates were determined using the BCA Protein Assay kit (Fisher Scientific, Loughborough, UK).

ELISA

Lysates of *L. donovani* DD8 (for Indian samples) or LEM3458 (for Sudanese samples), diluted to 2 μg/ml in 35 mM $NaHCO_3$/ 15 mM $NaCO_3$ buffer (pH 9.6), were added at 100 μl/well to Immulon 4HBX ELISA plates (VWR, Lutterworth, UK) and incubated overnight at 4°C. After washing the plates three times using PBS containing 0.05% (vol/vol) Tween 20 (Sigma, Gillingham, UK) (PBST), they were blocked using 200 μl/well PBST containing 2% skimmed milk powder (Premier International Foods, Spalding, UK) (PBSTM) at 37°C for 2 hr. Following three PBST washes, the human sera were added at 1:400 dilution for the Indian sera or 1:100 dilution for the Sudanese sera, in PBSTM, and the plates were incubated at 37°C for 1 hr. The different sample concentrations were used due to the significantly lower anti-*Leishmania* IgG response generated by the Sudanese VL patients, as recently described [13]. Following six PBST washes, 100 μl of horseradish peroxidase-conjugated subclass-specific mouse monoclonal antibodies specific for human IgG1 (ab99774), IgG2 (ab99784), IgG3 (ab99829) or IgG4 (ab99817) (Abcam, UK), diluted 1:5000 for the Indian sera or 1:1000 for the Sudanese sera, in PBSTM, were added and plates were incubated at 37°C for 1 hr.

Table 1. Single (unpaired) samples used in IgG subclass comparisons, and clinical status of the Indian and Sudanese patient groups.

	VL patient status					Controls		
Unpaired group	**Active**	**Cured**	**Relapsed**	**Treated[a]**	**PKDL**	**Other diseases**	**EHC (seropositive)**	**EHC (seronegative)**
Trial 1 India	20	21	19	-	-	20	-	20
Trial 2 (Expanded) India	46	28	35	-	24	28	28	32
Trial Sudan	47	-	-	22	23	-	30	12

EHC = endemic healthy control; PKDL = post kala-azar dermal leishmaniasis.
[a]treated, not in recent past, but time of treatment unknown.
doi:10.1371/journal.pntd.0003273.t001

Table 2. Paired and multiple sequential sera from Indian and Sudanese VL and PKDL.

	Country and disease status (VL or PKDL)							
Group and sampling	**India VL**			**India PKDL**				**Sudan VL**
Group	1	2	3	4	5	6	7	Sudan
Serum (days)	0 and 30	0 and 180	15 and 180	0–30	0–60	0–180	0–360	0 and 11, 17 or 30
Pairs/sequentials (seq)	24 pairs	32 pairs	43 pairs	2 seq	9 seq	9 seq	1 seq	17 pairs

Day 0 = before start of treatment.
doi:10.1371/journal.pntd.0003273.t002

Human IgG isotype responses were determined using 100 µl/well of a 1:5000 dilution for the Indian sera or 1:2500 dilution for the Sudanese sera, of horseradish peroxidase–conjugated donkey anti-human IgG secondary antibody (709-035-149: Jackson ImmunoResearch, West Grove, USA) with incubation at 37°C for 1 hr.

Following six PBST washes, 50 mM phosphate/citrate buffer (pH 5.0) containing 2 mM *o*-phenylenediamine HCl and 0.007% (vol/vol) H_2O_2 (Sigma, UK) was added at 100 µl/well and incubated in the dark at room temperature for 15 minutes. The substrate reactions were then stopped by the addition of 2M H_2SO_4 (50 µl/well) and the ELISA plates were read at 490 nm (Spectra Max 190, Molecular Devices, Sunnyvale, USA, or MRX II, Dynex Technologies, Chantilly, USA). These assays were performed on duplicate plates, simultaneously.

Statistical analyses

ELISAs cut-offs were determined by the mean values plus three standard deviations obtained from the seronegative endemic healthy control serum samples. Statistical analysis on ELISA data (2-tailed unpaired t-test for unpaired samples, and paired t-test for paired/sequential samples, with 95% confidence interval in both cases) was performed using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, California, USA).

Prototype *L. donovani* antigen-specific IgG1 immunochromatographic RDTs

The prototype lateral flow immunochromatographic tests consisted of a cassette with a nitrocellulose membrane, a sample pad, a conjugate pad and an absorbent pad, backed with a plastic strip. The nitrocellulose membranes were sensitized with crude *L. donovani* (strain LEM3458) antigen lysate produced as described above and anti-human IgG1-specific antibody labelled with colloidal gold was dried on the conjugate pad. This strip was housed in a plastic cassette with two windows: the application well and the test/reading window. Two prototypes of this assay were developed and tested. For prototype 1, 2 µl of serum and 2 µl of buffer were added to the nitrocellulose strip and then 90 µl of buffer was added to the application well. In prototype 2, the application of 2 µl of serum on the strip was followed by the addition of 75 µl of buffer in the application well. The human IgG that migrated over the nitrocellulose membrane reacted with the immobilized target antigens. The anti-human IgG1-specific conjugated MAbs rehydrated by the buffer recognised the antigen-bound human IgG1 in the sample, thereby resulting in a red-purple coloured band. A control line ensured that the human IgG and conjugated anti-human IgG1 migration had occurred successfully. The prototype tests were used with sets of post-chemotherapeutic sera from Indian patients considered to have relapsed VL (n = 30 for prototype 1, of which 22 were also used with prototype 2) or cured VL (n = 21 for prototype 1, of which 5 were also used with prototype 2). Fisher's exact 2-tailed test was used for testing the difference between proportions.

Results

The IgG1 subclass responses gave discrete separations between cured versus active VL and relapsed VL (Figure 1A and 1B). This potential human IgG1 subclass association with clinical VL status was therefore investigated further, in particular to determine whether it could be suitable for development as a rapid (lateral flow) diagnostic test (RDT) to detect relapsed VL patients at point of care (POC). IgG isotype responses showed a similar trend to IgG1; however as illustrated in Figure 2 (see below, paired

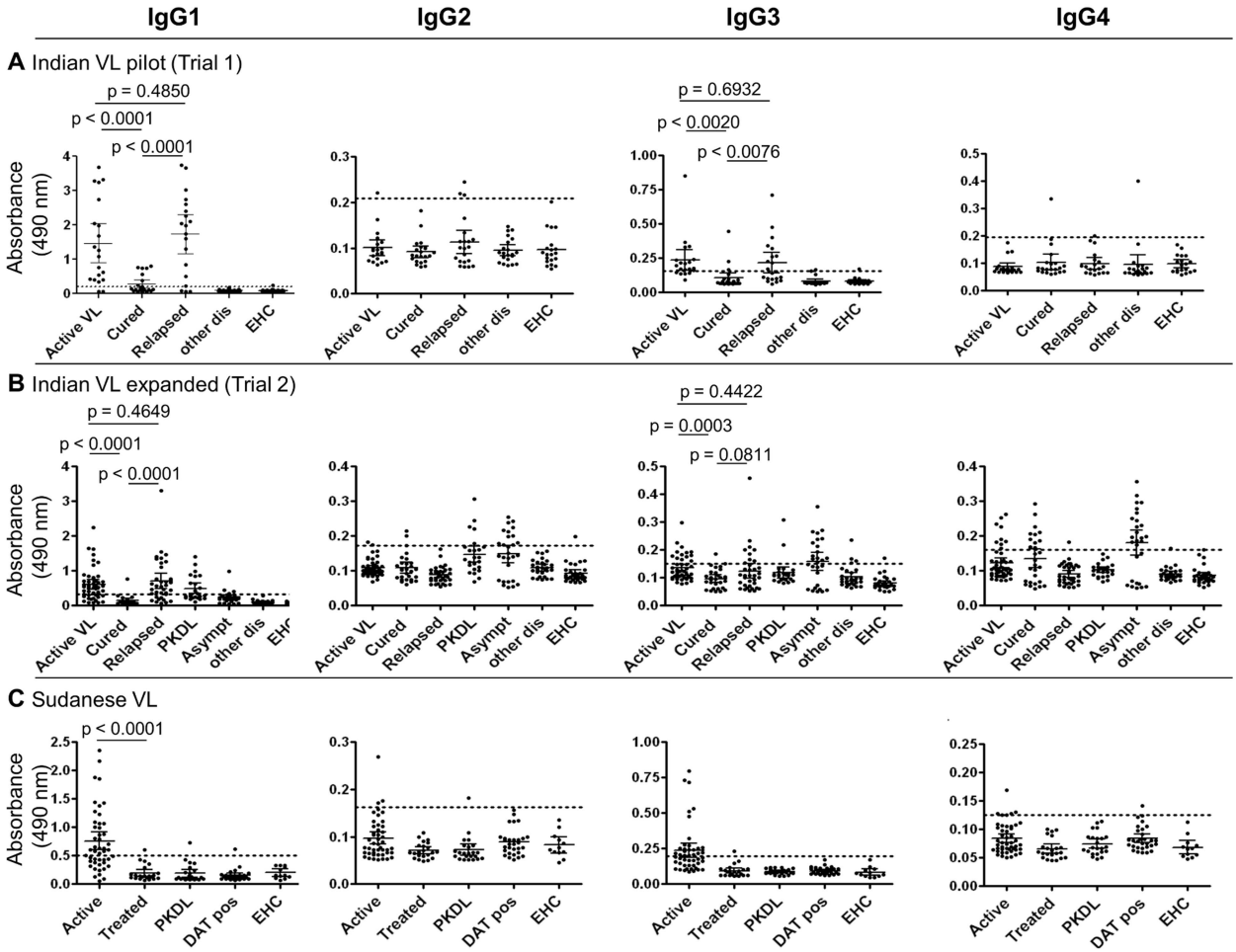

Figure 1. Specific IgG1 ELISA levels were high in active and relapsed VL but negative or substantially decreased in cured VL using unpaired serum samples. [A] Indian VL pilot study (Trial 1). [B] Indian VL expanded study (Trial 2). [C] Sudanese VL. Mean and 95% CI are shown for each data set (solid black lines); note the different Y axis scales. In each study set, the means plus three standard deviations obtained using DAT-seronegative endemic healthy control (EHC) samples was used to calculate the cut-off value (dotted line) and p values of <0.05 were considered significant.

doi:10.1371/journal.pntd.0003273.g001

samples) and already noted in the literature [33–37] this was less apparent and was inconsistent.

In Indian VL IgG1 levels were high pre-treatment and in relapse but were negative or substantially diminished in cured VL patients

Figure 1A and Table S1 show the results of the initial pilot study of IgG subclass in unpaired Indian VL patients with different clinical status. There was marked elevation of IgG1 levels in active (untreated) VL patients; IgG1 levels were dramatically reduced in patients who were treated and considered to be cured by clinical and parasitological assessments. Thus, 90.0% (18/20) pre-treatment active VL patients were serologically positive, above the cut-off value, whereas only 33.3% (7/22) patients considered to be cured remained positive, all of whom had low IgG1 titres ($p< 0.0001$, cured VL *versus* active or relapsed VL; Figure 1A). However, the IgG1 levels in the patients who were unsuccessfully treated were high and at levels comparable to the group of patients with active VL prior to treatment ($p = 0.485$; Figure 1A; Table S1). This trend for IgG1 levels was partially mirrored by IgG3. The IgG3 levels were however lower in the active VL patients (IgG1: 90.0% positive: IgG3: 75.0% positive) and the relapsed VL patients (IgG1: 84.2% positive: IgG3: 52.6% positive) patients and 47.4% (9/19) of these relapsed VL patients were IgG3 negative (Figure 1A; Table S1). In comparison IgG2 profiles were very weak (active VL: 5.0% positive; relapsed VL: 15.8% positive; cured VL: 0.0% positive) and IgG4 levels were negative or at the cut off boundary, except for one cured patient and one in the other disease group (a meningitis patient) (Figure 1A).

An expanded study of Indian VL confirmed that elevated IgG1 levels were a feature of relapse

Based on the initial study showing that elevated IgG levels were a potential biomarker of Indian VL treatment failure and relapse, a larger number of unpaired samples from Indian VL patients were analysed, together with those from a post kala-azar dermal leishmaniasis (PKDL) group, and from a group of patients who were VL asymptomatic but all of whom had positive DAT and/or rK39 serology. Since this second larger cohort was investigated after the initial study (Trial 1), the results are presented separately in Figure 1B, but were also incorporated into Table S1. In Trial 2 similar results were obtained to those in Trial 1, with 67.4%, 71.4% and 3.6% of the active VL, relapsed VL and cured VL

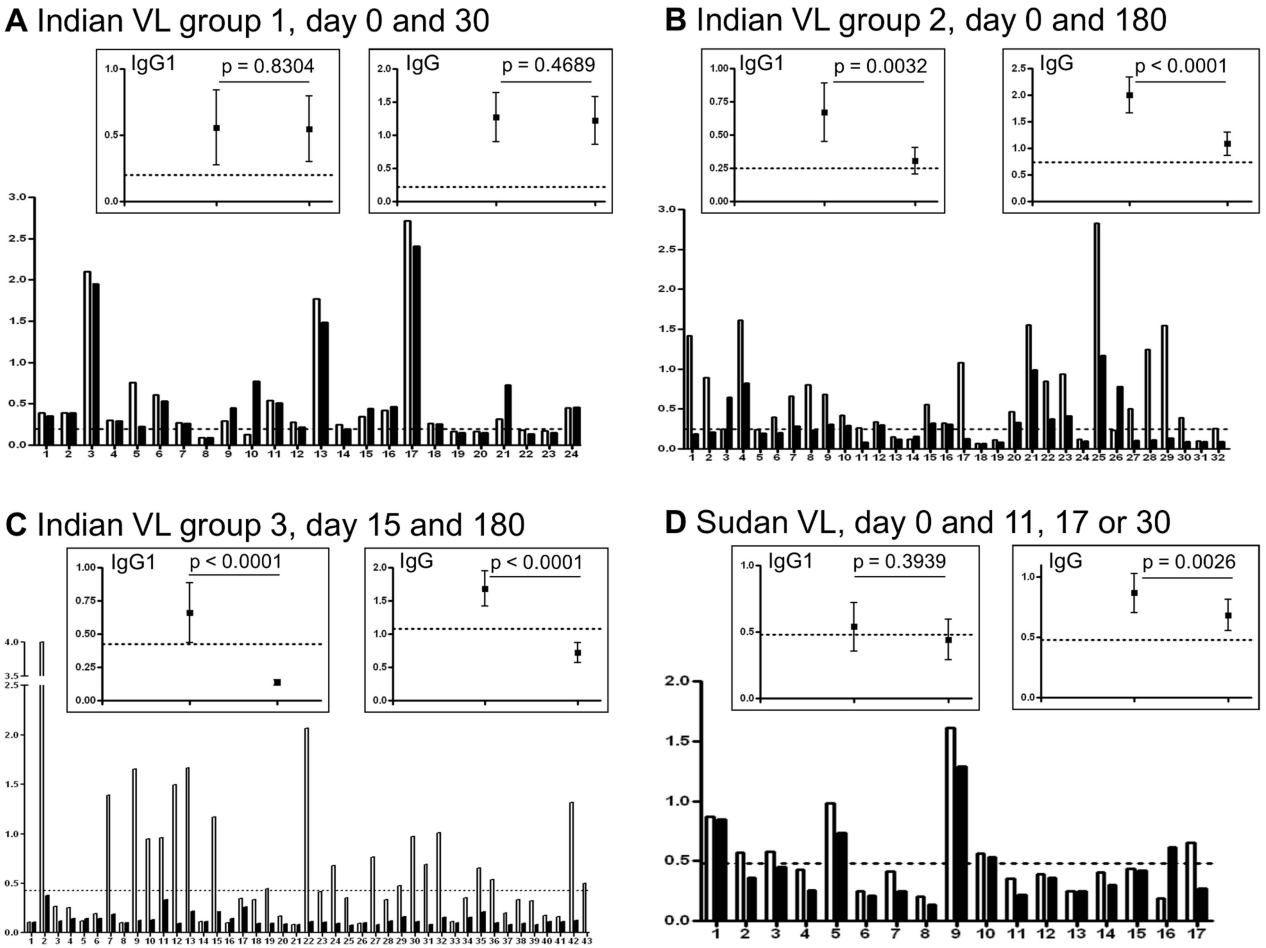

Figure 2. IgG1 decrease following cured VL became more significant with time. [A] Indian paired samples group 1, day 0 and 30. [B] Indian paired samples group 2, day 0 and 180. [C] Indian paired samples group 3, day 15 and 180. [D] Sudanese paired samples, day 0 and after treatment lasting 11, 17 or 30 days. Empty and filled columns represent the earlier and later samples of each pair respectively. Comparison of column heights allows the change in IgG1 level to be seen for each individual patient pre- and post–chemotherapy. For each data set represented in [A]–[D], paired samples from individual patients are presented in the main graph, and in the insets the mean and 95% CI compiled from all patients in that data set are shown for both IgG1 and IgG isotype. Mean plus three standard deviations of the results obtained using the seronegative endemic healthy control samples was used to calculate each cut-off value (dotted line) and p values of$<$0.05 were considered significant (subclasses IgG2–4 were also assayed but were not informative).
doi:10.1371/journal.pntd.0003273.g002

patients, respectively, being IgG1 positive (Figure 1B, Table S1). A lower percentage of the cured VL patients were IgG1 positive in Trial 2 compared to Trial 1 (Trial 1: 33.3% positive; Trial 2: 3.6% positive), although the absolute readings for Trial 1 cured were low and adjacent to cut-off borderline. IgG1 detection showed a much greater sensitivity and specificity than IgG3. Thus amongst the four IgG subclasses IgG1 was indicated for potentially identifying VL Indian patients with relapse versus cure after chemotherapy.

Sudanese VL patients had reduced IgG1 levels after treatment

Comparisons of Sudanese VL IgG subclass responses were performed with unpaired sera from active VL, treated VL and PKDL patients, as well as DAT-positive and DAT–negative endemic healthy controls (Table S1), at 1:100 dilution to allow for the overall lower IgG titres of Sudanese VL sera compared to Indian sera [13]. A slightly lower percentage of the Sudanese active VL patients (57.4%) had positive specific IgG1 levels compared to the Indian patients (Trial 1: 90.0%; Trial 2: 67.4%), but the treated Sudanese VL patients had low positive IgG1 levels (4.6%) similar to the cured VL patients in the Indian Trial 2 (3.6%). Thus the IgG1 levels in Sudanese active VL and Sudanese treated VL were significantly different ($p<0.0001$; Figure 1C, Table S1).

Anti-*L. donovani* IgG1 levels were raised in Indian PKDL

IgG1 levels were elevated in Indian PKDL compared to cured patients. Positivity rate was higher in Indian PKDL (Trial 2, 45.8%) than in Sudanese PKDL (4.3%), (Figures 1B and 1C, Table S1), although overall antibody levels in Sudanese VL were also lower, as we have reported previously [13].

In Indian VL, anti-*L. donovani* specific IgG1 levels decreased in paired samples approximately 6 months after successful treatment

Having determined that specific IgG1 levels were elevated in single serum samples from cases of VL treatment failure but were decreased in cured VL patients, we compared, for single individuals, paired serum samples obtained prior to or at start of

treatment and again at subsequent particular times. As shown in Table 2, for Indian VL paired samples from three Indian VL groups were analysed: group 1: day 0 and 30, group 2: day 0 and 180 and group 3: day 15 and 180. *L. donovani* antigen-specific IgG1 levels were not significantly reduced at 30 days after start of treatment (group 1: $p = 0.8304$; Figure 2A) but were significantly decreased at approximately 180 days after the start of therapy (group 2: $p = 0.0032$; group 3: $p<0.0001$; Figures 2B and 2C, respectively). In contrast, in Indian PKDL patients, a significant decrease in specific IgG1 levels was not observed using the sequential (day 0 to 30, 60, 180 or 360) samples (Table 2: groups 4–7).

In Sudanese VL specific IgG1 levels did not fall immediately after the start of treatment

Assay of IgG1 levels with paired samples from treated Sudanese VL patients (day 0 and 11, 17 or 30) showed that IgG1 subclass responses do not fall precipitously immediately after start of treatment (Figure 2D), as with Indian VL patients, which also showed no rapid fall in IgG1 level shortly after start of treatment (Figure 2A). However, with one exception, in which titre increased (patient 16, Figure 2D) all Sudanese VL patients showed a non-significant trend for titre to decrease slightly.

Prototype immunochromatographic rapid diagnostic tests could detect specific IgG1 levels indicative of relapsed VL

Two prototype immunochromatographic (lateral flow) RDTs were designed (see Methods) and tested to determine whether they could be used to discriminate between relapsed and cured VL patients by the detection of *L. donovani* antigen-specific IgG1 levels. The results for some of these patients are shown in Figure 3. As summarised in Table 3, most of the serum samples provided clear positive IgG1 results for the relapsed VL patients. Thus with prototype 1, 25 of 30 (83.3%) relapsed VL patients were positive but only 4 of 21 (19.0%) cured VL patients. Similarly, with prototype 2, 19 of 23 (82.6%) relapsed VL patients were positive but only 1 of 5 (20%) cured patients ($p<0.0001$ for the prototypes 1 and 2 cumulative results, Fisher's exact 2-tailed test). None of these 5 cured patients tested with either prototype 1 or 2 gave strong positive IgG1 signals. One of patients with malaria and no diagnosed VL had detectable IgG1. Thus, these prototype *L. donovani* antigen-specific IgG1 lateral flow assays could clearly discriminate between most of the relapsed and cured VL patients.

Discussion

Diagnosis of VL is not straightforward: clinical symptoms may overlap with other infectious diseases associated with fever syndrome; parasitological methods of diagnosis are invasive and have limited sensitivities. The currently recommended rK39-based rapid diagnostic tests have their limitations as they cannot distinguish systematically and reliably between the different clinical phases of VL. A large Indian/Nepalese population study reported an association between higher DAT and/or rK39 titres and risk of progression from asymptomatic to symptomatic VL [38]. Recent papers have studied asymptomatic (seropositive) populations in Bangladesh [39] and elsewhere [40–42]. In Bangladesh when assessing a patient cohort at 24 months follow-up for VL disease development, discrepancies were found between the molecular and serological tests [39]. In one long-term follow-up of 55 rK39 seropositive asymptomatic cases in India 69% developed VL and 31% remained asymptomatic [43]. However, the proportion of *L. donovani* complex seropositive asymptomatic individuals that progresses to symptomatic VL can be minor, and varies between endemic regions, for example between 1: 2.4 in Sudan [44], 4:1 in Kenya [45], 8:1 in Brazil [46], 4:1 in Bangladesh [47], 8.9:1 in India and Nepal [48], and 50:1 in Spain [49]. There is no rapid diagnostic test that determines which asymptomatic carriers will progress to active VL. Nor is there a rapid diagnostic test that is a biomarker of treatment failure and relapse as opposed to cure after chemotherapy. Thus development of such point of care tests has been identified by WHO as a research priority [8].

We are by no means the first to investigate the dynamics of antibody response in the different clinical phases and evolution of VL. The persistence of detectable anti-*Leishmania* IgG years after treatment, mainly using DAT and rK39, has been reported from India [33,34], Brazil [35], and Sudan [36,37]. We are not the first to mention that IgG subclass profiles may be associated with clinical status. The evaluation of IgG subclasses has been applied in other parasitic infections including echinococcosis [50–52], toxoplasmosis [53,54], and malaria [55–57]. Table 4 summarises

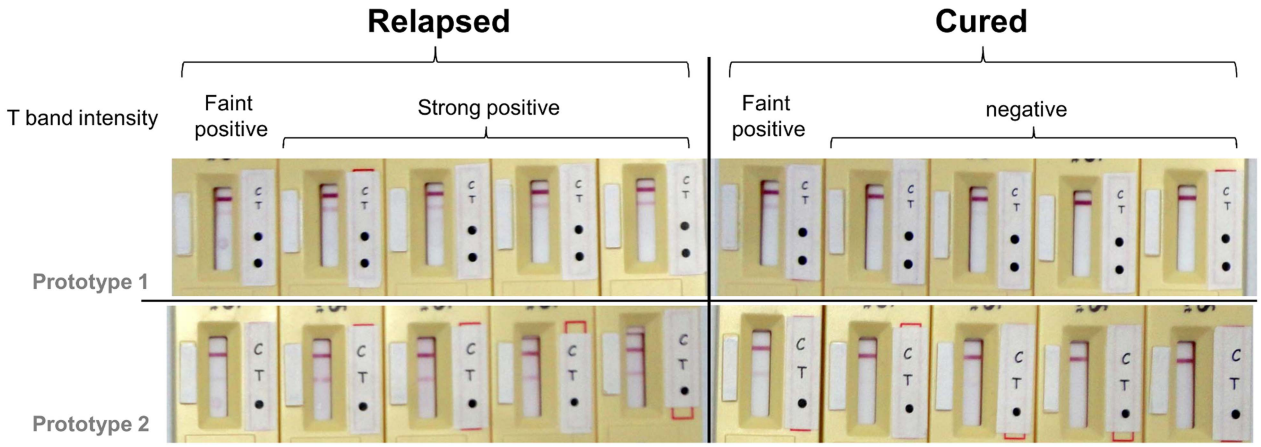

Figure 3. RDT IgG1 prototypes show the ability to distinguish relapsed VL from cured VL. C = migration control line; T = test line. Black dots indicate places where samples should be deposited: 2 µl serum in front of upper dot for prototype 1 or single dot in prototype 2, and 2 µl of buffer in front of lower dot in prototype 1.
doi:10.1371/journal.pntd.0003273.g003

Table 3. Summary of results from IgG1 rapid diagnostic tests (RDT) prototypes.

			RDT results	
Patient Groups	**n**	**RDT Used**	**Positive**	**Negative**
Relapsed VL[a]	30	Prototype 1	83.3% (25/30)	16.7% (5/30)
	23[b]	Prototype 2	82.6% (19/23)	17.4% (4/23)[c]
Cured VL	21	Prototype 1	19.0% (4/21)	81.0% (17/21)
	5[d]	Prototype 2	20.0% (1/5)	80.0% (4/5)[c]
Other diseases[e]	7	Prototype 1	14.3% (1/7)	85.7% (6/7)

[a]Therapy: sodium antimony gluconate n = 8; miltefosine n = 10; amphotericin B n = 3; combination therapy n = 2.
[b]22 of these samples were also used with prototype 1.
[c]these samples were also negative with prototype 1.
[d]these 5 samples were also used with prototype 1.
[e]malaria n = 3; hepatitis n = 1; TB n = 2; dengue n = 1.
doi:10.1371/journal.pntd.0003273.t003

previous published studies on IgG subclasses and clinical status of VL.

Here, using comparative plate ELISAs and in the context of previous literature, we have specifically examined the capacity of anti-*L. donovani* IgG subclass antibodies to act as a biomarker of therapeutic failure and relapse as opposed to cure. Furthermore, and most importantly, we have shown that the biomarker can be adapted to a lateral flow rapid diagnostic test suitable for use at point of care.

We analysed the IgG subclass profiles of patients in India with active VL and treated VL in comparison with asymptomatic seropositives, PKDL cases, other infectious diseases and endemic healthy controls. Future work could include a wider range of other diseases, including fungal infections. In a pilot study, we saw a remarkable decline in IgG1 levels in samples from unpaired Indian patients who were treated six months previously and were considered to be cured, so much so that the IgG1 titres for almost all of the individual patients fell below the ELISA cut off value for seropositivity (Figure 1A). IgG1 is produced in response to protein antigens and its decline with cure is thus presumably due to disappearance of the antigenic stimulus. A similar profile was to some extent also seen with IgG3, although IgG3 was not consistently raised above the cut-off in active VL. IgG2 levels were low across all groups (Figure 1). The results were similar for Sudan, in that there was a significantly lower level of IgG1 in non-recently treated patients, in almost all cases to below ELISA cut-off, compared to pre-treatment patients ($p<0.0001$; Figure 1C), although overall IgG and subclass titres were much lower for Sudanese active VL than Indian, as reported previously [13]. Other authors have referred to high IgG1 levels in active VL compared to healthy controls, and a decrease in IgG1 following successful therapy, as assessed by ELISA [17,21,23,29] or in one case by flow cytometry [30].

To explore the timing of the decline in IgG1 levels following successful chemotherapy of Indian VL we compared IgG1 ELISA titres prior to treatment, shortly after the start of treatment or approximately 180 days later, using paired Indian samples (groups 1–3, Table 2). At day 30 after start of treatment (group 1, Table 2) decline in IgG1 was minimal and not significant ($p = 0.8304$; Figure 2A), which is not surprising as the half life of human IgG1 is estimated to be around 21 days [58]. The slow decline in IgG1 shortly after treatment was confirmed with paired sera from treated Sudanese patients who had active VL ($p = 0.3939$; Table 2, Figure 2D).

We have not performed a western blot analysis with sera taken at different time points after treatment to determine whether the decrease in IgG1 titres relates to response to particular *L. donovani* antigens. However, one published study comparing western blot profiles using subclass specific conjugates and sera taken before and after treatment reported a general decline in band recognition and not the selective disappearance of bands [26]. Here we have used antigen derived from cultured promastigotes. However, in human VL the stage of the *Leishmania* life cycle is the amastigote, and given access to sufficient quantity of amastigote antigens it would be worthwhile to repeat such a comparative western blot study with amastigotes and subclass specific conjugates. In this way it might be possible to identify and subsequently isolate a specific amastigote antigen(s) applicable to determination of cure.

Recently decreases in IgG1 and IgG3 after cure [59] or post-active disease scarring [60] have also been reported for Brazilian cutaneous leishmaniasis (CL), and higher levels of these IgG subclasses in Turkish patients with active CL compared to endemic controls [61].

IgG subclass responses have been reported for experimental murine models of *Leishmania* infection and for canine infections with *L. infantum*, based on FcγR binding [62]. For canine leishmaniasis there are conflicting interpretations of IgG subclass profiles, (reviewed in [63]), reportedly due to confusion in subclass nomenclature of the commercial polyclonals used [63,64]. A recent study has proposed improvement of comparative studies by categorising canine IgG subclasses against function of their human analogues [65].

The detection of VL relapse following unsuccessful chemotherapy is of special importance because without effective treatment symptomatic VL is considered to be almost invariably fatal. Thus, if relapse is not recognized and followed up with repeated or alternative treatment, patients, who are often relatively isolated in rural endemic regions, will succumb to the disease. We were therefore interested to examine the IgG subclass profiles in unsuccessful treatment and relapse. Notably, IgG1 levels were raised in patients who failed to respond to chemotherapy and were considered to have relapsed 6 months after start of treatment. As in active VL the majority of relapsed patients had IgG1 titres clearly above the ELISA cut-off (Figures 1A and 1B). Such elevation of IgG1 in patients not responding to treatment has been mentioned rarely in the literature [21,23]. Accordingly, to provide an RDT for point of care application we devised two lateral flow

Table 4. IgG subclass serology in VL and PKDL: Published studies.

Reference	Origin of samples	Antigen (assay)	Authors' reports
[16]	Sudan	Intact promastigote (ELISA)	Elevated IgG1 and IgG3 in VL, not IgG2 or IgG4 (n = 15).
[17]	Sudan	Crude promastigote sonicate (ELISA)	Overall decrease in IgG1 and IgG3 (n = 28) 1 month post treatment of VL with sodium stibo-gluconate (Pentostam)
[18]	India	Crude promastigote sonicated lysate (ELISA); whole promastigote (immunoblotting)	IgG1>G2>G3>G4 in VL (n = 10) and PKDL (n = 6). IgG3 recognition of antigens by immunoblot persisted 24 weeks after successful chemotherapy, whereas IgG1 decreased in VL 24 weeks post chemotherapy.
[19]	Venezuela	Promastigote soluble extract (ELISA)	IgG1 predominant subclass; IgG4 also detectable (n = 10).
[20]	Somalia	Crude promastigote lysate (ELISA & western blot)	Elevated IgG1, IgG3, IgG4 in VL (n = 22).
[21]	India	Crude promastigote lysate (ELISA)	IgG1>IgG2>IgG3 = IgG4 before sodium antimony gluconate treatment in responders (n = 10) and non-responders (n = 10). 4–6 weeks post treatment, responders decreased all subclasses; non-responders no significant decrease.
[22]	India	Leishmanial membrane antigens (ELISA)	IgG1 predominant subclass, and IgG3 is useful diagnostic marker, in VL (n = 25).
[23]	India	Leishmanial membrane antigens (ELISA)	IgG1 increase in non-responders to sodium stibogluconate, reduced after subsequent cure by amphotericin B therapy (n = 5); all IgG subclasses decrease in sodium stibogluconate responders (n = 10)
[24]	Brazil, Colombia, Venezuela	Recombinant kinetoplastid membrane protein-1 (ELISA)	IgG1>>IgG3>IgG2>IgG4 in pre-treatment in VL (n = 12)
[25]	Ethiopia	Sonicated promastigote antigen (ELISA)	High IgG1 in VL (n = 10) compared to subclinical DAT positive (n = 18) and successfully treated (n = 20). IgG2 non-discriminating.
[26]	India	Leishmanial membrane antigens (western blot)	IgG1 50 days after sodium antimony gluconate therapy VL patients (n = 7) gave similar but less intense western blotting banding patterns.
[27]	India	Leishmanial membrane antigens (ELISA)	IgG1 elevated in PKDL (n = 23). IgG4 elevated in active VL (n = 10) but not in PKDL (n = 23). IgG1, IgG2, IgG3 overall higher in PKDL (n = 23) than in cured VL (n = 10)
[28]	India	Crude promastigote lysate (ELISA)	IgG1 elevated in VL (n = 38) compared to PKDL (n = 27); IgG2, IgG4 higher in PKDL than VL. IgG3 and IgG4 higher in paediatric (n = 16) than adult VL (n = 22). All IgG subclass levels comparable in paediatric (n = 7) and adult PKDL (n = 20)
[29]	India	Crude promastigote lysate (ELISA)	IgG1 and IgG3 decreased in VL 1 month post amphotericin B treatment (n = 6). Less IgG1 and IgG3 in macular (n = 5) than polymorphic PKDL (n = 11)
[30]	Brazil	Fixed *L. infantum* (syn. *L. chagasi*)promastigote (immunofluorescent flow cytometry)	IgG1>IgG3 in untreated VL (n = 21); absence of IgG2 and IgG4. IgG1 100% sensitive and specific for discriminating pre- and 12 month post-amphotericin B treatment paired sera of patients considered cured.
[31]	India	Crude promastigote lysate (ELISA)	IgG3>>IgG1>>IgG4>IgG2 in polymorphic PKDL (n = 3). IgG3>>IgG1>IgG2≥IgG4 in macular PKDL (n = 11) IgG1 and IgG3 decreased post treatment of polymorphic PKDL (n = 15)

doi:10.1371/journal.pntd.0003273.t004

prototypes, with which we assessed IgG1 seropositivity in treatment failure and relapse. Although we had already demonstrated that IgG1 levels were drastically reduced in cure, 6 months after treatment, for comparison we included a set of serum samples from such cured patients. Strikingly, the majority of patients considered to have relapsed were strongly positive in the IgG1 specific RDT, whereas none of the cured patients were strongly positive and the vast majority were entirely negative (Table 3, Figure 3). Further validation is indicated with a one year longitudinal study (since some patients may relapse between 6 months and one year after treatment [66]) and a larger cohort of patients, ideally with baseline application of the RDT prior to treatment, since despite successful treatment, extent of decline of IgG1 might be influenced by the pre-treatment titre. All relapse patients might be screened/rescreened for HIV infection to assess whether relapse may be associated with immunocompromised status. Thus, with optimisation and standardisation of reagents, and preferably with higher discriminative sensitivity, this RDT may provide an important and life-saving epidemiological tool to detect relapse of VL.

We have shown the potential of IgG1 to be a simple indicator of VL clinical status in terms of relapse. Symptomatic VL is treated at an acute phase of infection, which may favour the decline of IgG1 seropositivity in cure; it remains to be seen whether such markers are equally applicable to the treatment of prolonged chronic infections. As PKDL is a long-established chronic infection, this may explain why IgG1 does not decline with time after chemotherapy. A recent study has proposed that a high seropositivity in asymptomatic people may be associated with greater risk of progression to symptomatic VL [38]. Further work

could investigate whether high anti-*L. donovani* IgG1 titre in asymptomatic patients is a potential biomarker for progression to active VL.

Seroepidemiological comparisons with more detailed and sophisticated technological analysis of patient profiles is clearly a promising approach to defining precise, robust and widely applicable biomarkers.

Based on analysis and interpretation of our results, in conjunction with review of the relevant literature, we conclude that:

1. Retention of high anti-*L. donovani* IgG1 titre (or presumably rising titre) 6 months after chemotherapy (but not immediately after chemotherapy) is an indicator of treatment failure and relapse (however this is not likely to hold true for immunocompromised HIV co-infected patients, in whom antibody responses may be impeded). Patients with such a serological profile require follow-up and alternative treatment or will almost certainly succumb to fatal VL.
2. Most importantly we have shown that high anti-*L. donovani* IgG1 levels 6 months after treatment, and in comparison with preceding pre- or shortly post-treatment levels, are amenable to detection with a simple lateral flow POC applicable RDT. Nevertheless, such RDTs should not be applied at the exclusion of concomitant clinical evaluation.

Further investigation of seroepidemiological indicators is clearly justified to find even better alternative biomarkers of clinical status, in particular to identify asymptomatic progressors to VL, as well as to distinguish cure from treatment failure and relapse.

Supporting Information

Table S1 Single (unpaired) samples used in ELISA IgG subclass comparisons and clinical status of the Indian and Sudanese patient groups.
(DOCX)

Checklist S1 STROBE Checklist.
(DOCX)

Author Contributions

Conceived and designed the experiments: TB AKF MAM. Performed the experiments: TB AA MAG DEB OPS RK. Analyzed the data: TB AA MAG DEB MAM. Contributed reagents/materials/analysis tools: SS SES CT OPS RK OA OE AS PM. Wrote the paper: TB AKF OPS SSP MB PM MAM. Coordinated funding application and the NIDIAG associated research partnership: MB.

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Table S1. Single (unpaired) samples used in ELISA IgG subclass comparisons and clinical status of the Indian and Sudanese patient groups.

Patient group	n	IgG1		IgG2		IgG3		IgG4	
Trial 1 India		% positive	Mean A_{490} (95% CI)	% positive	Mean A_{490} (95% CI)	% positive	Mean A_{490} (95% CI)	% positive	Mean A_{490} (95% CI)
Active VL	20	90.0 (18/20)	1.47 (0.90 -2.03)	5.0 (1/20)	0.10 (0.08-0.12)	75.0 (15/20)	0.23 (0.16-0.31)	0.0 (0/20)	0.09 (0.07-0.10)
Cured VL	21	33.3 (7/21)	0.28 (0.15 – 0.40)	0.0 (0/21)	0.09 (0.08-0.10)	14.3 (3/21)	0.10 (0.06-0.14)	4.8 (1/21)	0.10 (0.7-0.13)
Relapsed VL	19	84.2 (16/19)	1.74 (1.17-2.31)	15.8 (3/19)	0.11 (0.09-0.14)	52.6 (10/19)	0.21 (0.14-0.29)	5.3 (1/19)	0.10 (0.08-0.12)
Other diseases	20	0.0 (0/20)	0.10 (0.08-0.11)	0.0 (0/20)	0.09 (0.08-0.11)	5.0 (1/20)	0.08 (0.07-0.09)	5.0 (1/20)	0.09 (0.06-0.13)
Endemic healthy control (seronegative)	20	5.0 (1/20)	0.09 (0.08-0.11)	0.0 (0/20)	0.10 (0.08-0.11)	5.0 (1/20)	0.08 (0.07-0.09)	0.0 (0/20)	0.10 (0.08-0.11)
Trial 2 (Expanded) India									
Active VL	46	67.4 (31/46)	0.61 (0.47-0.75)	4.3 (2/46)	0.11 (0.10-0.12)	28.3 (13/46)	0.13 (0.12-0.15)	40.0 (8/20)	0.12 (0.11-0.14)
Cured VL	28	3.6 (1/28)	0.15 (0.10-0.20)	7.1 (2/28)	0.11 (0.09-0.12)	3.6 (1/28)	0.10 (0.08-0.11)	35.7 (10/28)	0.14 (0.11-0.16)
Relapsed VL	35	71.4 (25/35)	0.70 (0.49-0.92)	0.0 (0/35)	0.09 (0.08-0.10)	25.7 (9/35)	0.12 (0.10-0.15)	2.9 (1/35)	0.09 (0.08-0.10)
post kala-azar dermal leishmaniasis	24	45.8 (11/24)	0.49 (0.34-0.64)	20.8 (5/24)	0.15 (0.12-0.17)	8.3 (2/24)	0.12 (0.10-0.14)	0.0 (0/24)	0.10 (0.09-0.11)
Asymptomatic (seropositive)	28	14.3 (4/28)	0.24 (0.17-0.31)	35.7 (10/28)	0.15 (0.12-0.17)	53.6 (15/28)	0.16 (0.13-0.19)	75.0 (21/28)	0.18 (0.14-0.22)
Other diseases	28	0.0 (0/28)	0.12 (0.10-0.14)	0.0 (0/28)	0.11 (0.10-0.12)	14.3 (4/28)	0.10 (0.09-0.12)	3.6 (1/28)	0.09 (0.08-0.10)
Endemic healthy control (seronegative)	32	3.1 (1/32)	0.12 (0.09-0.15)	3.1 (1/32)	0.09 (0.08-0.10)	3.1 (1/32)	0.08 (0.07-0.09)	3.1 (1/32)	0.09 (0.08-0.09)
Trial 1 Sudan									
Active VL	47	57.4 (27/47)	0.76 (0.60-0.92)	6.4 (3/47)	0.10 (0.08-0.11)	48.9 (23/47)	0.23 (0.19-0.28)	6.4 (3/47)	0.08 (0.08-0.09)
Treated VL[a]	22	4.6 (1/22)	0.19 (0.13-0.25)	0.0 (0/22)	0.07 (0.06-0.08)	4.6 (1/22)	0.09 (0.07-0.11)	0.0 (0/22)	0.07 (0.06-0.07)
post kala-azar dermal leishmaniasis	23	4.3 (1/23)	0.18 (0.11-0.25)	0.0 (0/23)	0.07 (0.06-0.08)	0.0 (0/23	0.09 (0.08-0.09)	0.0 (0/23)	0.07 (0.07-0.08)
DAT positive	30	3.3 (1/30)	0.15 (0.11-0.19)	0.0 (0/30)	0.09 (0.08-0.10)	0.0 (0/30)	0.09 (0.08-0.10)	3.3 (1/30)	0.08 (0.08-0.090
Endemic healthy control (seronegative)	12	0.0 (0/12)	0.20 (0.13-0.26)	0.0 (0/12)	0.08 (0.07-0.10)	0.0 (0/12)	0.08 (0.06-0.11)	0.0 (0/12)	0.07 (0.06-0.08)

[a] treated, not in recent past, but time of treatment unknown

In preparation

Costales J, Kotton C, Zurita-Leal A, Garcia-Perez J, Llewellyn M, Messenger L, Bhattacharyya T, Burleigh B. *Trypanosoma cruzi* I chronic chagasic cardiomyopathy and Chagas disease reactivation in Boston, Massachusetts, USA.

Candidate's contribution:

The candidate performed and analysed the *T. cruzi* lineage specific ELISA of the patient described in this case study.

ORAL PRESENTATION AND POSTERS

Oral presentations

WorldLeish5, Porto de Galinhas, Brazil, 2013. 'Understanding differential regional sensitivity of rapid diagnosis for visceral leishmaniasis'.

British Society for Parasitology Meeting, Bristol, 2013. 'Understanding differential regional sensitivity of rapid diagnosis for visceral leishmaniasis'.

Royal Society of Tropical Medicine & Hygiene Research-in-Progress, London, 2012. 'Extant cryptic sexuality in *Trypanosoma cruzi* drives the emergence of novel strains'.

American Society of Tropical Medicine & Hygiene Annual Meeting, Atlanta, USA, 2012. 'Towards *Trypanosoma cruzi* lineage-specific serology for Chagas disease'

British Society for Parasitology Meeting, Glasgow, 2012. 'Towards *Trypanosoma cruzi* lineage-specific serology for Chagas disease'

Royal Society of Tropical Medicine & Hygiene Research-in-Progress, London, 2011. 'Chagas disease serology: identifying infecting *Trypanosoma cruzi* lineages'.

British Society for Parasitology, Nottingham, 2011. 'Towards *Trypanosoma cruzi* lineage-specific serology for Chagas disease'

VII Workshop on Chagas Disease, CRESIB, Barcelona, Spain, 2011. 'Hacia la serología especifica para la identificación del linaje infectante de *T. cruzi*'.

XII International Congress of Parasitology (ICOPA), Melbourne, Australia, 2010. 'Using *Trypanosoma cruzi* lineage-specific synthetic peptides in Chagas disease serology'

Poster presentation – first author

British Society for Parasitology Symposium, Liverpool, 2013. 'Towards *Trypanosoma cruzi* lineage-specific serology for Chagas disease'

British Society for Parasitology Symposium, Liverpool, 2013. 'Understanding differential regional sensitivity of rapid diagnosis for visceral leishmaniasis'.

7th European Congress on Tropical Medicine & International Health, Barcelona, Spain, 2011. 'Genetic diversity and diagnostics development for trypanosomatid protozoa'.

Royal Society of Tropical Medicine & Hygiene Research-in-Progress, London, 2010. 'Chagas disease serology: identifying infecting *Trypanosoma cruzi* lineages'.

European Commission Colloquium**,** Paris, France 2010. 'A genomic approach to *Trypanosoma cruzi* lineage-specific serology for Chagas disease'.

British Society for Parasitology, Cardiff, 2010. 'A genomic approach to *Trypanosoma cruzi* lineage-specific serology for Chagas disease'.

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Co-author

Leiby D, Nguyen M, Bhattacharyya T, *et al*. **'**Preliminary investigations of a *Trypanosoma cruzi* lineage-specific serologic assay capable of identifying infected blood donors at greatest risk for transmitting infection' International Society of Blood Transfusion, Amsterdam, The Netherlands, 2013.

6. GENERAL DISCUSSION: RECAPITULATION AND FUTURE PERSPECTIVES

6.1 *Trypanosoma cruzi* and Chagas disease

From the first demonstration of the diversity of *T. cruzi* in naturally occurring cycles (Miles *et al*., 1977), the relevance to differing clinical and geographical presentations has been the subject of enquiry (Miles *et al*., 1981). Lineage identification, which was initially based on MLEE and then genotyping, requires that the parasite is first isolated. This is challenging in the post-acute phase of disease, due to the low parasitaemia, and confounded by the tissue tropisms of different lineages. Thus, the indirect approach of indentifying serologically antibodies produced against lineage-specific antigens has been applied here.

In this project, the *T. cruzi* surface mucin TSSA, previously described as being expressed on the bloodstream form of the parasite and exhibit sequence dimorphism (Di Noia *et al*., 2002), has been further investigated (ANNEX 1). By sequencing the part of the TSSA coding region containing the previously-described amino acid diversity from genomic DNA of isolates representing TcI-TcVI and spanning the geographical and ecological range of *T. cruzi*, a greater degree of TSSA diversity was identified. Specifically, the sequence reported as being restricted to isolates described grossly as 'TcII' (now called TcII-TcVI in subsequently revised nomenclature) was found only in TcII, TcV, and TcVI. In addition, TcV and TcVI also contained an alternate haplotype which differs by the substitution of an alanine residue in place of threonine. TcIII and TcIV were also both discovered to have their own lineage-specific sequences, not previousy identified. The TSSA sequence of TcI was found to be as previously reported (ANNEX 1: Figure 3).

Peptides based on these lineage-specific sequences were synthesised and used in this project in ELISA with serum from chagasic patients from a range of endemic countries in order to identify lineage-specific serological reactions (ANNEX 2). The use of recombinant, *E. coli* produced TSSA fusion proteins has been reported before (ANNEX 2: Table 3). In those studies, the TSSA sequences of the proteins contained not only the polymorphic region (residues 39-51), but also up to 26 of the flanking amino acids, which are highly conserved between *T. cruzi* lineages; however, this project is the first report of the use of synthetic peptides in this way. Here, recognition of the TSSApep-II/V/VI common peptide and

TSSApep-V/VI was very commonly found in serum originating from the Southern Cone countries (Brazil, Bolivia, Argentina), in agreement with their prevalence in this region based on MLEE/genotyping of parasite isolates. An unexpected finding was that 4/20 sera from Ecuador also reacted with TSSApep-II/V/VI, as these lineages had not previously been reported from that country. Another novel finding was the serological identification of TSSApep-IV, by one sample each from Colombia and Venezuela. However TSSApep-I, despite the broad geographical distribution of this lineage, was recognised only very rarely by the sera (ANNEX 2: Table 1 & Figure 3). The Colombian samples used here were the same as those used by Risso *et al.* (2011), in which western blotting of recombinant TSSA-II identified an unexpectedly high level of reactivity against this lineage north of the Amazon, including Colombia; however in this study, no such reactions were seen against the corresponding peptide TSSApep-II/V/VI.

Associations between peptide recognition and clinical symptoms were also investigated. Among the Brazilian patients, a much higher proportion of TSSApep-II/V/VI responders had ECG abnormailities than non-responders (38% vs. 17%, $p<0.0001$).

A novel bioinformatic analysis of the TSSA sequences from which these peptides derive predicted that TSSApep-II/V/VI and TSSApep-V/VI contain sequences of high antigenicity, whereas the TSSApep-I sequence predicts low antigenicity (ANNEX 2: Figure 4).

Thus this project has demonstrated that the greater polymorphism of the TSSA gene can be exploited in ELISA by using synthetic peptides based on this diversity, to identify an individual's history of lineage infection, association with cinical presentation, and geographical distribution of lineages.

Future perspectives & ongoing work:

Identification of a TcI-specific peptide: For unknown reasons, the TSSA peptide specific for TcI serology has not been successful in this project, even with those samples previously genotyped as containing TcI isolates, or presumed to do so due to geographical origin. Thus, identification of a robust TcI-specific peptide remains a crucial goal in sero-epidemiological studies of Chagas disease. The approaches to achieve this include using *in*

silico bioinformatic analyses on reference genomes to identify candidate proteins displaying desirable antigenic properties, and the use of genomic or synthetic peptide libraries in differential screening of TcI and TcII sera (natural or experimental).

Expanded studies of lineage identification and clinical symptoms: The link between infecting *T. cruzi* lineage(s) with the broad range of clinical symptoms remains largely circumstantial, with a paucity of candidate parasite or human factors identified. The application of lineage-specific serology to identify an individual's history of lineage infection would be a great aid in associating lineage to symptoms, and also potentially to act as a prognostic tool. This would require an expanded study of the type presented here (ANNEX 2), using samples from clinically well-characterised patients.

Adaptation to a lateral flow rapid test: TSSApep-II/V/VI, and to a lesser extent TSSApep-V/VI, have been shown here to be well recognised in the ELISA format for lineage-specific serology. A future and novel direction could be the incorporation of peptide(s) to a lateral flow, immunochromatographic, rapid diagnostic cassette, as described in Figures 13 and 32. This could be designed for use at point-of-care or in a research context, and also relevant for immunological surveillance of reservoir hosts (see following point).

Application to silvatic mammalian hosts: *T. cruzi* lineages circulate in the silvatic cycle by triatomine bugs feeding on mammal hosts. As with humans, lineage identification relies on parasite isolation and genotyping. The application of the lineage-specific serology described in this project would therefore have enormous potential in more fully understanding the ecology of lineages, particularly those which have been most associated with human disease. The use of TSSA (and future identified) lineage peptides, along with suitable host species-specific secondary antibodies, is envisaged to pursue this research direction.

6.2 *Leishmania donovani* and visceral leishmaniasis

Full-blown systemic, symptomatic VL is a gravely serious disease, which is almost always fatal without treatment. Accurate and timely diagnosis of infection with *L. donovani* complex is therefore of crucial importance in disease management. As shown in Tables 5 & 6, a significant difference in the sensitivity of the rK39 RDT between South Asia and East Africa had been previously identified. In this project, reasons potentially underlying this differential sensitivity were identified. These were parasite factors, namely the nature and extent of antigen diversity in the region coding for the 6.5 x 39 aa repeats used in rK39, and also human factors, namely the difference in overall anti-*Leishmania* IgG levels between VL patients from different endemic areas. Additionally, the nature of the IgG subclass response in VL patients of different status was analysed here.

This region coding for the 6.5 x 39 aa repeats used in rK39 was amplified and sequenced from a panel of East African *L. donovani* strains, and compared to published sequences (ANNEX 3). As expected from the fact that one of the PCR primers binds within a repeat region, multiple amplicons 117 bp apart were produced (ANNEX 3: Figure 2). On sequencing of the amplicon corresponding to 6.5 x 39 aa repeats from these strains, a wide range of polymorphism was found, including non-conservative charge changes, with the capacity to affect epitope structure and nature. In comparison with the sequence of rK39 and other published sequences mainly from South Asia, continent-specific polymorphisms were also identified (ANNEX 3: Figure 3). A similar approach to the proposed diagnostic antigen HASPB also revealed non-canonical combinations of repeat arrangements (ANNEX 3: Table 4).

As an alternative explanation for the lower sensitivity of rK39 RDT in East Africa, this project also analysed the role of anti-*Leishmania* IgG levels in VL patients from different endemic regions. These were Gedaref in Sudan and Bihar in India. Plasma samples from VL patients from both regions were reacted on the same ELISA plate coated with lysate antigen from both Indian and Sudanese *L. donovani* strains (ANNEX 4: Figure 1). Results were striking, with a significantly lower IgG response seen among Sudanese than Indian VL patients ($p<0.0001$). This was observed regardless of antigen source, age or gender of

patient (ANNEX 4: Figures 2&3). The lower IgG responses in the Sudanese cohort may be due to genetic, nutritional, or other factors.

In a WHO/TDR document (WHO, 2010e), case definitions for treatment outcome for VL patients were based on clinical and parasitological signs (Table 8), with another recent WHO publication listing an established marker of cure in VL as a knowledge gap and research priority (WHO, 2012). In this project, the discriminatory potential of IgG subclass responses in VL patients of differing clinical status by ELISA was evaluated (ANNEX 5). The clearest finding was the stark contrast in IgG1 levels comparing active (pre-treatment samples) plus those deemed to be therapeutically relapsed, versus those deemed to be therapeutically cured. The former group had displayed significantly elevated IgG1 levels compared to the latter ($p<0.0001$ for all cohorts of unpaired Indian and Sudanese samples; ANNEX 4: Figure 1). In accordance with the findings from the comparison of Sudanese and Indian overall anti-*Leishmania* IgG responses (Annex 4), the absolute values for the Sudanese IgG1 reactors were lower than for Indian. When performing the IgG1 ELISA on paired samples from the same patient before and either soon after or approximately 6 months after start of successful therapy, the IgG1 decrease in cured patients was manifest at the later time point when compared to the paired baseline samples (ANNEX 5: Figure 2). Following these findings, prototype immunochromatographic rapid tests to specifically detect IgG1 responses in VL patients were developed in collaboration with Coris BioConcept. Early results encourage the further, expanded evaluation of this test (ANNEX 5: Table 3 & Figure 3).

Future perspectives & ongoing work:

An expanded survey of the use of the IgG1 RDT: Following the encouraging early results on the utility of the prototype IgG1 RDT, it is envisaged to expand this to a larger set of samples, particularly paired samples of pre- and post therapy, those deemed to be either cured or relapsed. The RDT is also envisaged to be applied to asymptomatic patients who later progressed to symptomatic VL or remained as non-progressors, to investigate the utility of the IgG1 RDT as a tool for predicting disease progression, or more speculatively, on

the role of asymptomatc people in transmission to sandflies. The identification of further serological biomarkers is also encouraged by these results.

Identification of a *Leishmania* antigen indicative of current infection: Currently, non-parasitological diagnosis of VL depends on detection of anti-*Leishmania* IgG produced by the patient. The identification of *Leishmania* antigen(s) produced and excreted in the urine would be a non-invasive alternative for diagnosing active infection. An approach to this, as initiated by T Marlais, LSHTM, would involve the isolation of experimentally-derived anti-*Leishmania* IgG from immunised animals, and the subsequent generation of an anti-*Leishmania* IgG affinity column. After immunodepletion to remove common human urinary proteins from VL patients, any ligands which bound to the column would be eluted for analysis by mass spectroscopy to identify *Leishmania* protein fragments. Synthetic pepides or recombinant proteins derived from the parent molecules could then provide a basis for developing new serological or antigen capture assays.

6.3 Summary of overarching themes

This project has sought to analyse parasite diversity and improve serology for Chagas disease and visceral leishmaniasis. Both diseases are caused by protozoa of the order Kinetoplastida, family Trypanosomatidae, which share a number of morphological and molecular biological characteristics, but disparate modes of transmission and clinical disease. Consequently, they have been considered separately. Nevertheless, there are cetain common themes.

Molecular analysis of antigen diversity: Exploitation of *T. cruzi* TSSA diversity allows a deeper investigation of an individual's history of lineage infection, association with clinical symptoms, and geographical distribution. A similar approach to the *Leishmania* kinesin-like protein, a fragment of which is used as the rapid diagnostic antigen rK39, also revealed a great degree of diversity. However for VL, this diversity is predicted to disrupt the efficacy of serology using this antigen.

Diagnostic serology: In both Chagas disease and VL, a key research need identified by WHO is the development of biomarkers for disease status, as prognostic indicators or for monitoring treatment outcome. The research here has shown that elevated IgG1 levels in VL are associated with active disease or post-therapy relapse; subsequently, a similar serological survey of IgG subclass levels in Chagas disease was initiated. Early results, which

have not been included here, indicate that further investigations of these biomarkers in chagasic sera are also warranted.

Overall, for both *T. cruzi* and *L. donovani*, and for the serological reactions generated in their infected hosts, these results have shown the potential of deeper analyses to yield novel data and refined high resolution diagnostic and prognostic RDTs.

7. SUMMARY OF KEY FINDINGS

Trypanosoma cruzi and Chagas disease

- Greater genetic and amino acid diversity in the *Trypanosoma cruzi* surface mucin TSSA was revealed than had previously been reported, leading to the identification of lineage-specific epitopes [ANNEX 1 Bhattacharyya *et al*., 2010].
- Synthetic peptides based on TSSA diversity have been used to identify lineage-specific serological responses in chagasic patient sera, and for associating lineage-specifc reactions with geographical distributions and clinical symptoms [ANNEX 2 Bhattacharyya *et al*., 2014].

Leishmania donovani and visceral leishmaniasis

- A greater diversity in the *Leishmania donovani* diagnostic antigens rk39 and HASPB sequences has been demonstrated, with ample scope for affecting their diagnostic antigenicity. [ANNEX 3 Bhattacharyya *et al*., 2013].
- Comparative serology has shown that a Sudanese VL patient cohort generated a significantly weaker anti-*Leishmania* IgG response than a corresponding Indian cohort, revealing a possible cause of the differential RDT sensitivity in different geographical regions [ANNEX 4 Bhattacharyya *et al*., 2014].
- IgG1 levels were found to be elevated in active and relapsed VL patients, but substantially diminished in cured patients, over the course of several months post-therapy, and identification of this biomarker can be achieved by a novel prototype RDT [ANNEX 5 Bhattacharyya *et al*., 2014].

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APPENDIX

A.1 ChagasEpiNet Extracted from www.ki.se/chagasepinet/

"ChagasEpiNet is a European Union Seventh Framework Program funded project whose aim is to elucidate the epidemiology of the genetic lineages of *Trypanosoma cruzi* for improved understanding and prevention of Chagas disease.This project unites compatible research skills across 15 partner institutions in Europe and South America to study the disease in different endemic areas.

- Improve and standardise techniques for genotyping *T. cruzi*.
- Improve our understanding of the population genetics of *T. cruzi*
- Sequence the genome of *T. cruzi* lineage I.
- Develop lineage specific immunological diagnosis.
- Pilot studies to evaluate *T. cruzi* lineage specific associations with 1) Clinical outcome; 2) Congenital infection; 3) Cellular infectivity; & 4) Susceptibility to trypanocidal drugs."

INSTITUTE	**LOCATION**
LSHTM	London, UK
Universidad Mayor de San Simon	Cochabamba, BOLIVIA
Universidad Central de Venezuela	Caracas, VENEZUELA
Instituut voor Tropische Geneeskunde	Antwerpen, BELGIUM
Universidad Nacional de Salta	Salta, ARGENTINA
Université Libre de Bruxelles	Brussels, BELGIUM
Fundação Oswaldo Cruz	Rio de Janeiro, BRAZIL
GEMI-IDR	Montpellier, FRANCE
Universidade Federal de Goiás	Goiania, BRAZIL
Karolinska Institutet	Stockholm, SWEDEN
Universidad de los Andes	Bogota, COLOMBIA
University of East Anglia	Norwich, UK
Universidad de Chile	Santiago, CHILE
Universidad Autónoma de Madrid	Madrid, SPAIN
Pontificia Universidad Católica del Ecuador	Quito, ECUADOR

A.2 NIDIAG Extracted from www.nidiag.org

"NIDIAG is a European research network (Collaborative Project) coordinated by the Institute of Tropical Medicine. Our consortium brings together 13 complementary partners, including 2 private companies and 11 laboratories, from 4 European Member States and Associated Countries, 4 countries in Asia and 3 countries in Africa. The project is supported by the European Commission under the Health Priority of the 7th Framework Programme. The aim of the NIDIAG project is improve the quality of NID care at primary health care level in resource-poor settings. The major objectives of the NIDIAG consortium are: to develop and validate an integrated syndromic approach based on diagnosis treatment algorithms for three NID-related clinical syndromes frequently encountered in primary care settings,

namely the persistent fever syndrome (at least 2 week duration), the neurological syndrome, and the intestinal syndrome; to develop novel diagnostic platforms/assays tailored to specific epidemiological contexts at primary care level in NID-endemic settings"

INSTITUTE	LOCATION
Instituut voor Tropische Geneeskunde	Antwerpen, BELGIUM
LSHTM	London, UK
Inserm-Transfert	Paris, FRANCE
Université de Genève	Geneva, SWITZERLAND
Institut National de Recherches Biomédicales	Kinshasa, DR CONGO
Gadjah Mada University	Yogyakarta, INDONESIA
Institut National de Recherche en Santé Publique	Bamako, MALI
Swiss Tropical Institute	Basel, SWITZERLAND
Banaras Hindu University	Varanasi, INDIA
B.P. Koirala Institute of Health Sciences	Dharan, NEPAL
University of Khartoum	Khartoum, SUDAN
Sihanouk Hospital Center of HOPE	Phnom Penh, CAMBODIA
Coris BioConcept	Gembloux, BELGIUM

A.3 Public websites and online databases

GenBank	http://www.ncbi.nlm.nih.gov/genbank/
NCBI BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi
TriTrypDB	http://tritrypdb.org/tritrypdb/

A.4 Molecular biology reagents

1 x NEBuffer 2 (NEB):	50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithothreitol (pH 7.9 @ 25°C).
1 x NH_4 buffer (Bioline):	16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 @ 25°C), 0.01% stabilizer.
1 x TAE buffer:	40 mM Tris, 1 mM EDTA, 20 mM glacial acetic acid (prepare 50x stock).
DNA loading buffer ('home-made'):	1 x TAE buffer, 40% (v/v) glycerol, bromophenol blue, in ddH_2O

Reagent/consumable/apparatus	Supplier	Cat. No.
0.2 ml strip tubes & caps	VWR	732-0551
Agarose	Bioline	BIO-41025
Ampicillin	Sigma	A0166
Big Dye Terminator v3.1	Applied Biosystems	433 7455
BioTaq DNA polymerase	Bioline	BIO-21040
Bromophenol blue	Sigma	114405
Dilution buffer (DNA sequencing)	Applied Biosystems	4336697
DNA loading buffer (commercial)	Bioline	BIO-37045
DNEasy Blood & Tissue kit	Qiagen	69504
dNTPs (10mM mix)	NEB	N0447S
EDTA	Sigma	E5134
EtBr	VWR	443022U
EtOH, absolute	Fisher	E/0650DF/17
Glacial acetic acid	VWR	100015N
Hyperladder I	Bioline	BIO-33025
Hyperladder IV	Bioline	BIO-33029
Hyperladder V	Bioline	BIO-33031
IPTG	Bioline	BIO-37036
isopropanol	VWR	20842.323
NEBuffer 2	NEB	B7002S
NH_4 buffer	Bioline	BIO-37025
PTC-100 & -200 Thermal cycler machines	MJ Research	
Puregene Tissue Core Kit Cell lysis solution, *Proteinase K, *RNaseA, Protein precipitation solution, DNA hydration buffer. * = available separately: Proteinase K - 158918 RNAseA – 158922	Qiagen	158667
*Pvu*II	Promega	R6331
QIAquick Gel Extraction Kit	Qiagen	28704
QIAquick PCR Purification Kit	Qiagen	28104
QIAGEN Spin miniprep kit	Qiagen	27106
SureClean reagent	Bioline	BIO-37042
Tris (TRIZMA base)	Sigma	T6066
UV transilluminator	UVP	95-0216-02
X-gal	Bioline	BIO-37035

A.5 GenBank submissions

T. cruzi

WHO CODE OF STRAIN	**Lineage**	**TSSA** (ANNEX 1)	**RNA binding protein** (ANNEX 2)
MDID/US/00/92101601P cl1	TcI	GU059926	
MPRC/US/00/92122102R	TcIV	GU075672	
MHOM/BO/00/92:80	TcV		KJ395485
IINF/BR/00/Bug2148 cl1	TcV	GU059934	KJ395486
MHOM/BR/00/CanIII cl1	TcIV	GU075671	
MHOM/CL/00/CBB cl3	TcII	GU059929	KJ395479
IINF/PY/00/Chaco9	TcVI		KJ395491
IINF/PY/00/Chaco17	TcVI		KJ395490
IINF/PY/00/Chaco23	TcII		KJ395481
MINF/BR/00/CL Brener	TcVI	GU075678	KJ395488
MDAS/CO/00/CM17	TcIII	GU075674	
MPHT/BO/00/COTMA47	TcI		KJ395476
MDAS/BR/00/Cutia cl1	TcI	GU059922	
MCAN/US/00/Dog Theis	TcIV	GU059927	
IINF/AR/00/EPV20-1	TcVI		KJ395492
MHOM/BR/00/Esm cl3	TcII	GU075675	
MHOM/CL/00/IVV cl4	TcII	GU059931	KJ395477
IINF/AR/00/LHVA	TcIV		KJ395483
MDAS/BR/00/M5631 cl5	TcIII	GU075673	
MHOM/BR/00/M6241 cl6	TcIII	GU059932	
MHOM/BR/00/MAS cl1	TcII	GU059930	
MHOM/CL/00/MN cl2	TcV	GU075677	
MHOM/VE/00/OPS21 cl11	TcI	GU059925	
MHOM/BO/00/P209 cl93	TcI	GU059924	
MHOM/BO/00/P251	TcVI		KJ395489
IINF/PY/00/P63 cl1	TcVI	GU059936	
IINF/PY/00/Para6	TcV		KJ395487
MHOM/PE/00/SaxP18	TcI		KJ395472
IINF/BO/00/SC43 cl1	TcV	GU075676	
MHOM/BR/78/Sylvio-X10/1	TcI	GU059921	KJ395471
MDID/BO/2004/SJM37	TcI		KJ395475
MPHI/BO/2004/SJM41	TcI		KJ395473
MDID/BO/00/SMA2	TcI		KJ395474
IINF/BO/00/SO3 cl5	TcV	GU059935	
IINF/CL/00/Sp104 cl1	TcI	GU059923	
IINF/PY/00/T655	TcII		KJ395482
IINF/BO/00/Tu18 cl93	TcII	GU059928	KJ395480
MHOM/CL.00/Tula cl2	TcVI	GU059937	
IINF/CL/00/VFRA	TcVI		KJ395493
IINF/CL/00/Vinch101	TcV		KJ395484
MCAN/PY/00/X109/2	TcIII	GU059933	
MHOM/BR/00/Y	TcII		KJ395478

[a] WHO code: MHOM/BR/78/Sylvio-X/10
Elements: 1 2 3 4 5

[1] Type of host or vector from which isolated: M =Mammalia; I = Insecta. [2] Generic name of host or vector: AKO = *Akodon boliviensis*; CAN = *Canis familiaris*; DAS = *Dasypus* sp.; DID = *Dideplhis* spp.; HOM = *Homo sapiens*; INF = *Triatoma infestans*; PHI = *Philander* sp.; PHT = *Phylottis* sp.; PRC = *Procyon* sp. [3] Country of isolation, in accordance with International Organisation for Standardization: AR = Argentina; BO = Bolivia; BR = Brazil; CL = Chile; CO = Colombia; PE = Peru; PY = Paraguay; US = United States of America; VE = Venezuela. [4] Year of isolation: Last two digits of year starting 19__ ; all four digits of years starting 20__ ; 00 = unknown. [5] Laboratory designation.

L. donovani ANNEX 3

		HASPB		
WHO CODE OF STRAIN [a]	**kinesin**	**HASPB1**	**HASPB2**	**unpredicted**
MHOM/ET/72/GEBRE 1		KC342850		
MHOM/SD/82/GILANI	KC342869	KC342851	KC342856	
MHOM/ET/67/HU3 (LV9)	KC342866	KC342849	KC342855	
MHOM/ET/00/HUSSEN	KC342867			KC342861
MHOM/SD/97/LEM3458		KC342854	KC342860	
MCAN/SD/98/LEM3556		KC342853	KC342859	
MHOM/SD/98/LEM3582	KC342870	KC342852	KC342857	
IMAR/KE/62/LRC-L57	KC342872			KC342863
MHOM/KE/67/MRC(L)3				KC342864
MHOM/SD/00/SUDAN1	KC342871		KC342858	
MCAN/IQ/81/SUKKAR 2				KC342865
MHOM/SD/87/UGX-marrow	KC342868			KC342862

[a] WHO code: MHOM/ET/72/GEBRE 1
Elements: 1 2 3 4 5

[1] Type of host or vector from which isolated: M =Mammalia; I = Insecta; [2] Generic name of host or vector: CAN = *Canis familiaris*; HOM = *Homo sapiens*; MAR = *Phlebotomus martini;* [3] Country of isolation, in accordance with International Organisation for Standardization: ET = Ethiopia; IQ = Iraq; KE = Kenya; SD = Sudan; [4] Year of isolation: Last two digits of year starting 19__ ; 00 = unknown; [5] Laboratory designation.

A.6 ELISA reagents

1 x Coating buffer: 15 mM Na_2CO_3, 34 mM $NaHCO_3$. (prepare 10x stock, store 4°C).

1x PBS (pH7.4): 137 mM NaCl, 8 mM Na_2HPO_4 (anhydrous), 2.7 mM KCl, 1.5 mM KH_2PO_4

Wash buffer: PBS, 0.05% Tween-20.

Blocking buffer: PBS, 2% Skimmed milk powder, 0.05% Tween-20.

Substrate solution (prepared immediately before use):

10ml phosphate citrate buffer pH5.5

(approx. 1:1 ratio of 100 mM Citric acid : 100 mM Na_2HPO_4)

4 mg O-phenylenediamine HCl ($[C_6H_8N_2.2HCl]$; 2 mM final)

3ul 30% H_2O_2

Stop solution: 2M H_2SO_4

Reagent/consumable	**Supplier**	**Cat. No.**
Adhesive plate seals	Sigma	A5596 or G676001
Avidin (resuspended to 1mg/ml in PBS)	Sigma	A9275
BCA Protein assay kit	Fisher	PN23227
Citric acid monohydrate	VWR Sigma	10081 C1909
H_2O_2	VWR Sigma	23615.261 216763
H_2SO_4	VWR	20700.265
Immulon 4HBX 96w flat bottomed plate	VWR	735-0465
KCl	VWR	101984L
KH_2PO_4	VWR	102032W
NaCl	Sigma	S7653
Na_2CO_3	Sigma	S6139
$NaHCO_3$	Sigma	S5761
Na_2HPO_4 anhydrous	VWR	301584L
$Na_2HPO_4.12H_2O$	VWR	102484A
O-phenylenediamine HCl	Sigma	P1526
PBS (tablets)	Sigma VWR Fisher	P4417 524 650-1 10209252
Reagent reservoir	VWR	613-1176
Skimmed milk powder	-	Brand 'Marvel'
Streptavidin-HRP conjugate	Sigma	S2438
Spectrophotometer Helios Epsilon	Fisher	SPR-200-150B
Tween-20	Sigma	P7949

A.7 HRP-conjugated secondary antibodies (anti-human)

Target	Raised in	pAb/mAb	Supplier	Cat. No.
IgG (H+L)	donkey	polyclonal	Stratech	709-035-149
IgG1 (γ chain)	mouse	monoclonal	Abcam	ab99774
IgG2 (γ chain)	mouse	monoclonal	Abcam	ab99784
IgG3 (hinge H Chain)	mouse	monoclonal	Abcam	ab99829
IgG4 γ chain	mouse	monoclonal	Abcam	ab99817

A.8 Cell culture reagents

A.8.1 *T. cruzi*

Stock solutions

1M HEPES		23.8g/100 ml H_2O, pH 7.2	
Tryptone/trypticase		17.5g/100 ml H_2O	
Haemin		250mg/100 ml 0.01M NaOH	
PGAB		Final	100x stock
	Na Glutamate	2 mM	3.4g/100 ml
	Na Pyruvate	2 mM	2.2g/ 100 ml
	Gentamycin	50 µg/ml	500 mg/ml

To 500ml RPMI 1640, add 50ml heat-inactivated FBS.

Combine in a 50ml tube the stock solutions: 10 ml HEPES

14 ml Tryptone

4 ml Haemin

6 ml PGAB

Filter this mix through a 0.2 µm filter directly into the RPMI/FBS. Store at 4°C.

A.8.2 *Leishmania*

αMEM

i) To 800ml ddH_2O in a 1 L bottle or beaker, dissolve:

		(final)
aMEM powder	1bottle	(1x)
NaHCO3	2.2g	(26.2 mM)
L-glutamine	0.59g	(4 mM)
D-glucose	3g	(16.7 mM)
HEPES	10g	(42 mM)

Adjust pH to ~7.5. Add ddH_2O to 860 ml.

ii) Prepare:

Haemin	5mg /50 µl 1N NaOH/ 9.95 ml dH_2O	(0.008 mM)
Adenine	40 mg /500 µl 1N NaOH /9.50 ml dH_2O	(0.3 mM)
Folic acid	5mg /40 µl 1N NaOH/ 9.96 ml dH_2O	(0.011 mM)
D-biotin	2mg /100 µl 1N NaOH /9.90 ml dH_2O	(0.008 mM)

iii) Transfer aMEM made to this point into a clean 1L bottle. In a cell culture cabinet, add:
Haemin, Adenine, Folic acid, D-biotin solutions;
gentamycin 1 ml (50 ug/ml)

iv) Sterile filter into an autoclaved 1 L bottle, using a bottle-top filter and vacuum pump.

v) Add
Foetal Calf serum (heat inactivated) 100 ml (10%)

vi) Store at 4 °C.

Reagent/consumable	Supplier	Cat. No.
αMEM powder	Sigma	M0644
24 well plate	VWR	734-0020
48 wel plate	VWR	734-0028
Adenine	Sigma	A2786
D-Biotin	Sigma	B4639
FBS	Sigma	F9665
Folic acid	Sigma	F8758
Gentamycin	Sigma	G1522
D-Glucose	Sigma	G8270
L-Glutamine	Sigma	G8540
Haemin	Sigma	H5533
HEPES	Sigma	H3784
Na Glutamate	Sigma	49621
Na Pyruvate	Sigma	P5280
$NaHCO_3$	Sigma	S5761
NaOH	Sigma	S5881
RPMI	Sigma	R0883
Tryptone/trypticase	Sigma	T9410
FastRead 102 disposable counting chamber	ImmuneSystems	BVS100
Parafilm	VWR	291-1212
Slope tube	Fisher	TKT-150-050B
Bottle-top filter	Fisher	TKV-240-015F
T25 culture flask	SLS	353014
T75 culture flask	SLS	353024
Vacuum pump	Fisher	FB70155

A.9 List of suppliers

Aapptec	www.aapptec.com
Abcam	www.abcam.com
Abgene	www.abgene.com
Advanced Chromatography Technologies	www.ace-hplc.com
Agilent Technologies	www.genomics.agilent.com
Applied Biosystems	www3.appliedbiosystems.com
Bioline	www.bioline.com
Bio-Rad	www3.bio-rad.com
Chembio Diagnostics	chembio.com
Dynex	www.dynextechnologies.com
Eppendorf	www.eppendorf.co.uk

Eurofins MWG Operon	www.eurofinsdna.com
Fisher Scientific	www.fisher.co.uk
Genosphere Biotechnologies	www.genosphere.co.uk
Jackson Labs	*see* Stratech
Merck	www.merck-chemicals.co.uk
MJ Research	www.mj-research.com
MSE (Measuring and Scientific Equipment)	www.mseuk.co.uk
NEB (New England Biolabs)	www.neb.uk.com
Oxoid	www.oxoid.com
Promega	www.promega.com
Rathburn Chemicals Ltd	www.rathburn.co.uk
Qiagen	www1.qiagen.com
Sigma-Aldrich	www.sigmaaldrich.com
SLS (Scientific Laboratory Supplies)	www.scientificlabs.co.uk
Stratech (*for Jackson Labs)*	www.stratech.co.uk
Syngene	www.syngene.com
UVP	www.uvp.com
VWR	uk.vwr.com
Zinsser Analytic	www.zinsser-analytic.com